09/762224

Copy f r the Elected Offic (EO/US)

PCT/US99/17702

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SCHWARTZ, Jason, J.
Woodard, Emhardt, Naughton,
Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 21 February 2001 (21.02.01)	
Applicant's or agent's file reference 7024403	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/17702	**International filing date** (day/month/year) 04 August 1999 (04.08.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
PURDUE RESEARCH FOUNDATION Office of Technology Transfer 1063 Hovde Hall West Lafayette, IN 47907 United States of America	US	US
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
PURDUE RESEARCH FOUNDATION Office of Technology Commercialization 1291 Cumberland Avenue West Lafayette, IN 47906 United States of America	US	US
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer A. Karkachi
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

003852117

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 21 September 2000 (21.09.00)	
International application No. PCT/US99/17702	**Applicant's or agent's file reference** 7024403
International filing date (day/month/year) 04 August 1999 (04.08.99)	**Priority date** (day/month/year) 04 August 1998 (04.08.98)
Applicant SANDERS, David, A. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

01 March 2000 (01.03.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Manu Berrod
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

REVISED VERSION

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17702

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12P 21/06; C12N 7/04, 5/00; A61K 39/12
US CL :435/69.1, 236, 325; 424/199.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 236, 325; 424/199.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPATFUL, MEDLINE, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,739,018 A (MIYANOHARA et al.) 14 April, 1998, see entire document.	1-55
Y	US 5,512,421 A (BURNS et al.) 30 April 1996, see entire document.	1-55
Y	US 5,591,624 A (BARBER et al.) 07 January 1997, see entire document.	1-55
Y	US 5,503,974 A (GRUBER et al.) 02 April 1996, see entire document.	1-55
Y	US 5,723,287 A (RUSSELL et al.) 03 March 1998, see entire document.	1-55
Y	US 5,278,056 A (BANK et al.) 11 January 1994, see entire document.	1-55

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
25 JANUARY 2000

Date of mailing of the international search report
01 SEP 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
Jeffrey S. Parkin, Ph.D.
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:

SCHWARTZ, Jason, J.
Woodard, Emhardt, Naughton,
Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ETATS-UNIS D'AMERIQUE

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

Date of mailing (day/month/year) 21 September 2000 (21.09.00)	
Applicant's or agent's file reference 7024403	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US99/17702	04 August 1999 (04.08.99)	04 August 1998 (04.08.98)

Applicant
PURDUE RESEARCH FOUNDATION et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SL,SZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AE,AL,AM,AT,AZ,BA,BB,BR,BY,CH,CR,CU,DK,EE,ES,FI,GB,GD,GE,GH,GM, HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MW,MX,PT,SD,SG,SI, SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
Manu Berrod

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:

SCHWARTZ, Jason, J.
Woodard, Emhardt, Naughton,
Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ÉTATS-UNIS D'AMÉRIQUE

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 01 October 1999 (01.10.99)	
Applicant's or agent's file reference 7024403	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/17702	International filing date (day/month/year) 04 August 1999 (04.08.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 04 August 1998 (04.08.98)
Applicant PURDUE RESEARCH FOUNDATION et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
04 Augu 1998 (04.08.98)	60/095,242	US	24 Sept 1999 (24.09.99)
15 Dece 1998 (15.12.98)	60/112,405	US	24 Sept 1999 (24.09.99)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Carlos Naranjo

Telephone No. (41-22) 338.83.38

WO 00/08131
PCT/US99/17702

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
SCHWARTZ, Jason, J.
Woodard, Emhardt, Naughton,
Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 17 February 2000 (17.02.00)	
Applicant's or agent's file reference 7024403	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US99/17702	04 August 1999 (04.08.99)	04 August 1998 (04.08.98)

Applicant
PURDUE RESEARCH FOUNDATION et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,EP,IL,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CR,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GD,GE,GH,GM,
   HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,
   RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   17 February 2000 (17.02.00) under No. WO 00/08131

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

DEC 22 1999

PCT

From the INTERNATIONAL BUREAU

To:

SCHWARTZ, Jason, J.
Woodard, Emhardt, Naughton,
Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ÉTATS-UNIS D'AMÉRIQUE

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

Date of mailing (day/month/year)
14 December 1999 (14.12.99)

Applicant's or agent's file reference
7024403

IMPORTANT NOTIFICATION

International application No.
PCT/US99/17702

International filing date (day/month/year)
04 August 1999 (04.08.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
NORTH, Cynthia, Lin
3131 Thomas Drive
Lafayette, IN 47905
United States of America

State of Nationality: US
State of Residence: US
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
NORTH, Cynthia, Lin
3803 B Sickle Court
Lafayette, IN 47905
United States of America

State of Nationality: US
State of Residence: US
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[X] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[ ] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
V. Gross

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:

SCHWARTZ, Jason, J.
Woodard, Emhardt, Naughton,
Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ÉTATS-UNIS D'AMÉRIQUE

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

Date of mailing (day/month/year) 14 December 1999 (14.12.99)	
Applicant's or agent's file reference 7024403	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/17702	**International filing date** (day/month/year) 04 August 1999 (04.08.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
SHARKEY, Curtis, Matthew Apartment 6 319 North 5th Street Lafayette, IN 47904 United States of America	US	US
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
SHARKEY, Curtis, Matthew Apartment 6 1307 Columbia Lafayette, IN 47901 United States of America	US	US
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[X] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[ ] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer V. Gross
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PCT INTERNATIONAL APPLICATION TRANSMITTAL LETTER	DATE 04 August 1999
REGARDING THE INTERNATIONAL APPLICATION OF PURDUE RESEARCH FOUNDATION, etal.	DOCKET OR REFERENCE NUMBER 7024403
ENTITLED PSEUDOTYPED RETROVIRUSES AND STABLE CELL LINES FOR THEIR PRODUCTION	

09/762224

JC02 Rec'd PCT/PTO 02 FEB 2001

Certification under 37 CFR 1.10 (if applicable)

EL016469485US
"Express Mail" mailing number

04 August 1999
Date of Deposit

I hereby certify that this application is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Linda S. W. Conrad
(Typed or printed name of person mailing application)

Linda S.W. Conrad
(Signature of person mailing application)

To the United States Receiving Office (RO/US):

Accompanying this transmittal letter is the above-identified International application, including a completed Request form (PCT/RO/101). Please process the application according to the provisions of the Patent Cooperation Treaty.

The following requests are made of the RO/US:

1. [XX] PREPARATION AND TRANSMITTAL OF CERTIFIED COPY OF PRIORITY DOCUMENTS—Please prepare and transmit to the International Bureau a certified copy of the United States origin priority documents identified in Box VI of the Request form (37 CFR 1.451).

   To cover the cost of copy preparation and certification (37 CFR 1.19(a)(3) and (b)(1)),
   [XX] a (check) (money order) in the amount of $ 30.00 included in fee is attached to this transmittal letter.
   [ ] the RO/US is hereby authorized to charge the following deposit account no.: _____.

2. [XX] CHOICE OF INTERNATIONAL SEARCHING AUTHORITY—It is requested that the International Search be performed by the following International Searching Authority:
   [XX] United States Patent and Trademark Office (ISA/US)
   [ ] European Patent Office (ISA/EP)

   The appropriate Search fee for the above-named Authority is indicated on the Fee Calculation Sheet (PCT/RO/101 Annex).

3. [XX] SUPPLEMENTAL SEARCH FEES (ONLY WHEN ISA/US CONDUCTS THE INTERNATIONAL SEARCH.)—Please charge any Supplemental Search fees that may be required by the United States International Searching Authority (ISA/US) to deposit account no.: 23-3030.

   I understand that this authorization is subject to my oral confirmation thereof in each instance and that it in no way limits my right to submit a protest against payment of the Supplemental Search fees, but is merely an administrative aid to assure that the ISA/US may timely complete the Search Report.

NOTE: SUPPLEMENTAL SEARCH FEES FOR ISA/EP ARE PAYABLE DIRECTLY TO THE EUROPEAN PATENT OFFICE

4. [XX] DISCLOSURE INFORMATION—In order to assist in screening the accompanying International application for purposes of determining whether a license for foreign transmittal should and could be granted and for other purposes, the following information is supplied:

   A. [ ] There is no prior filed application relating to this invention.

   B. [XX] There ~~is~~ are a prior application, serial number 60/095,242 ~~60/112,405~~ filed on 04 August 1998 (04.08.98) ~~15 December 1998 (15.12.98)~~ which contains subject matter that is

      1. [ ] substantially identical to that of the accompanying International application.
      2. [X] less than that of the accompanying International application. The additional subject matter of the International application appears on pages(s) and line(s) throughout the application
      3. [ ] more than that of the accompanying International application.

   C. [ ] Disclosure information cannot be covered by the language of Points 4A or 4B above due to the involvement of several prior applications or for other reasons. A separate sheet on which the disclosure information is explained is attached to this transmittal letter.

5. [XX] REQUEST FOR FOREIGN TRANSMITTAL LICENSE—According to the provisions of 35 U.S.C. 184 and 37 CFR 5.11, a license to transmit the accompanying International application to foreign agencies or international authorities is hereby requested.

SIGNER IS THE	NAME OF SIGNER (typed)
[ ] APPLICANT [ ] COMMON REPRESENTATIVE [X] (ATTORNEY) (AGENT) REG NO 43,910	Jason J. SCHWARTZ SIGNATURE Jason J. Schwartz

PTO-1382 (REV 3-84)
USCOMM-DC 84-3817

U.S. Department of Commerce
Patent and Trademark Office

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference: 7024403

Applicant: PURDUE RESEARCH FOUNDATION, etal.

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . 240 [T]

2. SEARCH FEE . . . . . 700 [S]

International search to be carried out by US

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 70 sheets.

first 30 sheets . . . . . 455 [b1]

40 (remaining sheets) x 10 (additional amount) = 400 [b2]

Add amounts entered at b1 and b2 and enter total at B . . . . 855 [B]

Designation Fees

The international application contains 80 designations.

10 (number of designation fees payable *(maximum 10)*) x 105 (amount of designation fee) = max. 1050 [D]

Add amounts entered at B and D and enter total at I . . . . . 1905 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . 30 [P]

5. TOTAL FEES PAYABLE . . . . . 2875.00 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

XX	authorization to charge deposit account (see below)	☐	bank draft	☐	coupons
XX	cheque	☐	cash	☐	other *(specify)*:
☐	postal money order	☐	revenue stamps		

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ US

☐ is hereby authorized to charge the total fees indicated above to my deposit account.

XX *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

23-3030 (Deposit Account No.) 04/08/99 (Date *(day/month/year)*) Signature: Jason A. SCHWARTZ, #43,910

Form PCT/RO/101 (Annex) (January 1999; reprint July 1999)

See Notes to the fee calculation sheet

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For Receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 7024403

Box No. I TITLE OF INVENTION PSEUDOTYPED RETROVIRUSES AND STABLE CELL LINES FOR THEIR PRODUCTION

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

PURDUE RESEARCH FOUNDATION
Office of Technology Transfer
1063 Hovde Hall
West Lafayette, Indiana 47907 US

☐ This person is also inventor.

Telephone No. 765-494-2610

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SANDERS, David A.
324 Jefferson Drive
West Lafayette, Indiana 47906 US

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SCHWARTZ, Jason J.
WOODARD, EMHARDT, NAUGHTON, MORIARTY & MCNETT
Bank One Center/Tower, Suite 3700
111 Monument Circle
Indianapolis, Indiana 46204 US

SEE CONTINUATION TO BOX NO. IV ON SHEET NO. 5

Telephone No. 317-634-3456

Facsimile No. 317-637-7561

Teleprinter No. 810-341-3283

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the follow[illegible] sub-boxes is used, this sheet should not be in[illegible]ed in the request.

Name and address: *(Family n[illegible] followed by given name; for a legal entity, full official designation. The address mu[illegible]de postal code and name of country. The country of address indicated in this Box [illegible]pplicant's State (that is, country) of residence if no St[illegible] of residence is indicated below.)*

KUHN, Richard John
7501 Amanda Lane
West Lafayette, Indiana 47906
United States of America

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

JEFFERS, Scott A.
1945 Indian Trail Drive
West Lafayette, Indiana 47906 US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SHARKEY, Curtis Matthew
319 North 5th Street, Apt. 6
Lafayette, Indiana 47904 US

This person is.
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

NORTH, Cynthia Lin
3131 Thomas Drive
Lafayette, Indiana 47905 US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Continuation of Box No. III FUR R APPLICANT(S) AND/OR (FURTHER) IN TOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

FISCHBACH, Michael A.
120 Pathway Lane
West Lafayette, Indiana 47906 US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is.
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-box(es); at least one must be marked)*:

Regional Patent

- [X] **AP** **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- [X] **EA** **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- [X] **EP** **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [X] **OA** **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- [X] **AE** United Arab Emirates
- [X] **AL** Albania
- [X] **AM** Armenia
- [X] **AT** Austria
- [X] **AU** Australia
- [X] **AZ** Azerbaijan
- [X] **BA** Bosnia and Herzegovina
- [X] **BB** Barbados
- [X] **BG** Bulgaria
- [X] **BR** Brazil
- [X] **BY** Belarus
- [X] **CA** Canada
- [X] **CH and LI** Switzerland and Liechtenstein
- [X] **CN** China
- [X] **CU** Cuba
- [X] **CZ** Czech Republic
- [X] **DE** Germany
- [X] **DK** Denmark
- [X] **EE** Estonia
- [X] **ES** Spain
- [X] **FI** Finland
- [X] **GB** United Kingdom
- [X] **GD** Grenada
- [X] **GE** Georgia
- [X] **GH** Ghana
- [X] **GM** Gambia
- [X] **HR** Croatia
- [X] **HU** Hungary
- [X] **ID** Indonesia
- [X] **IL** Israel
- [X] **IN** India
- [X] **IS** Iceland
- [X] **JP** Japan
- [X] **KE** Kenya
- [X] **KG** Kyrgyzstan
- [X] **KP** Democratic People's Republic of Korea
- [X] **KR** Republic of Korea
- [X] **KZ** Kazakhstan
- [X] **LC** Saint Lucia
- [X] **LK** Sri Lanka
- [X] **LR** Liberia
- [X] **LS** Lesotho
- [X] **LT** Lithuania
- [X] **LU** Luxembourg
- [X] **LV** Latvia
- [X] **MD** Republic of Moldova
- [X] **MG** Madagascar
- [X] **MK** The former Yugoslav Republic of Macedonia
- [X] **MN** Mongolia
- [X] **MW** Malawi
- [X] **MX** Mexico
- [X] **NO** Norway
- [X] **NZ** New Zealand
- [X] **PL** Poland
- [X] **PT** Portugal
- [X] **RO** Romania
- [X] **RU** Russian Federation
- [X] **SD** Sudan
- [X] **SE** Sweden
- [X] **SG** Singapore
- [X] **SI** Slovenia
- [X] **SK** Slovakia
- [X] **SL** Sierra Leone
- [X] **TJ** Tajikistan
- [X] **TM** Turkmenistan
- [X] **TR** Turkey
- [X] **TT** Trinidad and Tobago
- [X] **UA** Ukraine
- [X] **UG** Uganda
- [X] **US** United States of America
- [X] **UZ** Uzbekistan
- [X] **VN** Viet Nam
- [X] **YU** Yugoslavia
- [X] **ZA** South Africa
- [X] **ZW** Zimbabwe

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

- [X] CR – Costa Rica
- [ ] .....
- [ ] .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental B x *If the Supplemental Box is not used, this sheet should not be included in the request.*

*1. If, in any of the Boxes, **the space is insufficient** to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America**: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the nam of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box No. IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "**patent of addition**," or "**certificate of addition**," or if, in Box No. V, the name of the United States of America is accompanied by an indication "**continuation**" or "**continuation-in-part**": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed**: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, **the earlier application is an ARIPO application**: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.*

Continuation to Box No. IV Agent

WOODARD, Harold R.; EMHARDT, C. David; NAUGHTON, Joseph A., Jr.; MORIARTY, John V.; McNETT, John C.; HENRY, Thomas Q.; DURLACHER, James M.; REEVES, Charles R.; WAGNER, Vincent O.; ZLATOS, Steve; BEREVESKOS, Spiro; BAHRET, William F.; BROWNING, Clifford W.; FRISK, R. Randall; LUEDERS, Daniel J.; GANDY, Kenneth A.; THOMAS, Timothy N.; SISSELMAN, Kerry P.; JONES, Kurt N.; ALLIE, John H.; BANTA, Holiday W.; COLE, Troy J.; PAYNTER, L. Scott; LOWES, J. Andrew; MEYER, Charles J.; HARRIS, Darrin Wesley; SCHANTZ, Matthew R.; COY, Gregory B.; HIDAY, Lisa A.; DANILUCK, John V.; BROWN, Christopher A.;SCHWARTZ, Jason J.; USHER, Arthur J. IV; COLLIER, Douglas A.; MYERS, James B. Jr.; STEVENS, Scott J., and ROWE, James L., all of Woodard, Emhardt, Naughton, Moriarty & McNett, Bank One Center/Tower, Suite 3700, 111 Monument Circle, Indianapolis, Indiana 46204 United States of America

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) (04.08.98) 04 August 1998	60/095,242	US		
item (2) (15.12.98) 15 December 1998	60/112,405	US		
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): (1), (2)

** Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)* :

ISA / US

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date (day/month/year)	Number	Country (or regional Office)
04 August 1998 (04.08.98)	60/095,242	US
15 December 1998 (15.12.98)	60/112,405	US

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	:	6
description (excluding sequence listing part)	:	42
claims	:	11
abstract	:	1
drawings	:	4
sequence listing part of description	:	6
Total number of sheets	:	70

This international application is **accompanied** by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: Transmittal Letter (dup)

Figure of the drawings which should accompany the abstract: —

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Applicant(s):
PURDUE RESEARCH FOUNDATION;
SANDERS, David A.; KUHN, Richard John;
JEFFERS, Scott A.; SHARKEY, Curtis Matthew;
NORTH, Cynthia Lin; FISCHBACH, Michael A.

Agent:

(Jason J. SCHWARTZ)

For receiving Office use only

1. Date of actual receipt of the purported international application:	2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority (if two or more are competent): ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

TOC 115

RECEIVED

SEP 19 2000

Woodard, Emhardt, Naughton, Moriarty & McNett

PATENT COOPERATION TREA

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:
JASON J. SCHWARTZ
WOODARD, EMHARDT, NAUGHTON, MORIARTY
& MCNETT, BANK ONE CENTER/TOWER
111 MONUMENT CIRCLE, SUITE 3700
INDIANAPOLIS, IN 46204

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Date of mailing *(day/month/year,*	14 SEP 2000
Applicant's or agent's file reference: 7024403	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/17702	04 AUG 99	04 AUG 98

Applicant
PURDUE RESEARCH FOUNDATION

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:
01 March 2000 (01-03-00)

2. That date of receipt is:

- [x] the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
- [ ] the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
- [ ] the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. [ ] **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

- [ ] *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on: _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/ Assistant Commissioner for Patent	Authorized officer
Box PCT Washington, D.C. 20231 Attn:RO/US Facsimile No. 703-305-3230	Telephone No. 703-305-3677 703.308-6454

Form PCT/IPEA/402 (July 1998)

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: JASON J. SCHWARTZ
WOODARD, EMHARDT, NAUGHTON, MORIARTY, & MCNETT
BANK ONE CENTER/TOWER, SUITE 3700
111 MONUMENT CIRCLE
INDIANAPOLIS, INDIANA 46204

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	03 JAN 2001
Applicant's or agent's file reference: 7024403	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US99/17702	04 AUGUST 1999	04 AUGUST 1998

Applicant
PURDUE RESEARCH FOUNDATION

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	S. Parkin
Facsimile No. (703) 305-3230	Telephone No. (703) 308-1234

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 7024403	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/17702	International filing date *(day/month/year)* 04 AUGUST 1999	Priority date *(day/month/year)* 04 AUGUST 1998
International Patent Classification (IPC) or national classification and IPC IPC(7): C12P 21/06; C12N 7/04, 5/00; A61K 39/12 and US Cl.: 435/69.1, 236, 325; 424/199.1		
Applicant PURDUE RESEARCH FOUNDATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV [X] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [ ] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
01 MARCH 2000	27 NOVEMBER 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer J. S. Parkin Telephone No. (703) 308-1234

Form PCT/IPEA/409 (cover sheet) (July 1998)★

PATENT COOPERATION TREATY

09/762224

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 7024403	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/17702	International filing date *(day/month/year)* 04 AUGUST 1999	Priority date *(day/month/year)* 04 AUGUST 1998
International Patent Classification (IPC) or national classification and IPC IPC(7): C12P 21/06; C12N 7/04, 5/00; A61K 39/12 and US Cl.: 435/69.1, 236, 325; 424/199.1		
Applicant PURDUE RESEARCH FOUNDATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
01 MARCH 2000	27 NOVEMBER 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer J. S. Parkin Telephone No. (703) 308-1234

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/17702

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-42 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 43-53 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-6 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/17702

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:

Please See Supplemental Sheet.

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

   ☒ all parts.

   ☐ the parts relating to claims Nos. _.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/17702

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-55	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-55	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-55	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-55 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the claimed invention. The claimed invention is directed, *inter alia*, toward cells that are capable of producing retroviruses pseudotyped with glycoproteins derived from different filoviruses or cells that are capable of producing pseudotyped retroviral particles comprising at least two different viral envelope glycoproteins. Additional aspects of the invention encompass methods of producing said cells, the pseudotyped particles themselves, methods of using said particles, and kits useful for the production of said particles. Although the prior art discloses the preparation of pseudotyped retroviral vector particles (RVVPs), it suffers from a number of limitations. For instance, the RVVPs of the prior art suffer from a number of limitations including limited host range, cellular toxicity during particle expression due to envelope glycoprotein toxicities, unstable particle formation, and low RVVP titers. Accordingly, there is a need in the art for pseudotyped RVVPs with a broad host range and cell lines that are capable of producing the same. Applicants have addressed this need by providing eukaryotic cells that either transiently or stably produce pseudotyped RVVPs comprising at least two different viral glycoproteins in their lipid bilayer. The inclusion of filoviral envelope glycoproteins into said RVVPs is one particularly preferred embodiment. The pseudotyped RVVPs of the instant invention are useful in transducing host cells of interest, display a reduced cellular toxicity, have a broad host range, and reduce pseudotransduction events.

---------- NEW CITATIONS ----------

NONE

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

IV. LACK OF UNITY OF INVENTION:

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2, and 13.3 is not complied with for the following reasons:

Group I, claims 1-12, drawn to a eukaryotic cell comprising nucleotide sequences encoding, inter alia, at least two different viral glycoproteins.
Group II, claims 13-18, drawn to a eukaryotic cell comprising nucleotide sequences encoding, inter alia, a filoviral glycoprotein.
Group III, claims 19-29, drawn to a method of producing a eukaryotic cell capable of producing pseudotyped retroviruses with two different viral glycoproteins.
Group IV, claims 30-32, drawn to a method of producing a eukaryotic cell capable of producing pseudotyped retroviruses with a filoviral glycoprotein.
Group V, claims 33-38, drawn to a pseudotyped retrovirus containing at least two different viral glycoproteins.
Group VI, claim 39, drawn to a pseudotyped retrovirus containing a Marburg virus glycoprotein.
Group VII, claims 40-43, drawn to a method of introducing a nucleotide sequence into a cell by transducing a cell with a pseudotyped retrovirus expressing at least two different viral glycoproteins.
Group VIII, claim 44, drawn to a method of introducing a nucleotide sequence into a cell by transducing a cell with a pseudotyped retrovirus expressing a Marburg virus glycoprotein.
Group IX, claims 45-49 and 51, drawn to a method of screening for agents effective in blocking viral entry employing a pseudotyped retrovirus expressing at least two different viral glycoproteins.
Group X, claims 50, 52, and 54, drawn to a method of screening agents effective in blocking Marburg virus entry into a cell employing a pseudotyped retrovirus expressing a Marburg virus glycoprotein.
Group XI, claim 53, drawn to a kit for forming pseudotyped retroviruses containing at least two different viral glycoproteins.
Group XII, claim 55, drawn to a kit for forming psuedotyped retroviruses containing a Marburg virus glycoprotein.

The inventions listed as Groups I-XII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The claims are directed toward multiple products (e.g., eukaryotic cells, pseudotyped retroviral particles, kits) with different chemical structures/compositions and attendant features (e.g., expressing two different viral glycoproteins, expressing a single virus glycoprotein). The claims are also directed toward multiple methods (e.g., method of making a eukaryotic cell capable of producing retroviral pseudotypes, method of gene transduction employing pseudotyped retroviral particles, method of screening for putative antiviral agents) that employ different reagents, methodology steps, and accomplish different scientific objectives. Accordingly, the claims all lack a special technical feature and are directed toward different inventive concepts.

CORRECTED VERSION*

PCT

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(54) Title: PSEUDOTYPED RETROVIRUSES AND STABLE CELL LINES FOR THEIR PRODUCTION

(57) Abstract

Cells that produce inventive pseudotyped retroviruses having a broad host range have been produced. In one aspect of the invention, the cells produce retroviruses pseudotyped with at least two different viral glycoproteins, such as togaviral glycoproteins. In alternative embodiments, the cells produce retroviruses pseudotyped with filoviral glycoproteins. Methods of producing the above-described cells, as well as the pseudotyped retroviruses thus produced, are also provided. In other embodiments, methods of screening agents effective in blocking viral entry into a cell, including filoviral entry or entry of viruses having at least two different viral glycoproteins disposed in their lipid bilayer, such as togaviruses, are provided. Moreover, methods of using the inventive pseudotyped retroviruses for introducing nucleotide sequences into target cells, and kits for forming the inventive pseudotyped retroviruses, are also provided.

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PSEUDOTYPED RETROVIRUSES AND STABLE CELL LINES FOR THEIR PRODUCTION

REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Patent Application Serial Number 60/095,242, filed on August 4, 1998, and U.S. Patent Application Serial Number 60/112,405, filed on December 15, 1998, which are both hereby incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

The present invention relates generally to cells that produce pseudotyped retroviruses having broad host range. Specifically, the invention relates to cells that produce retroviruses pseudotyped with glycoproteins derived from either filoviruses or viruses having at least two different viral glycoproteins disposed in their lipid bilayer. The invention further relates to methods of producing such cells, the pseudotyped retroviruses produced, methods of making and using the pseudotyped retroviruses and kits for producing the pseudotyped retroviruses.

Retroviruses are ribonucleic acid (RNA) viruses that include an RNA genome enclosed within a viral capsid wherein the capsid is surrounded by an envelope, or lipid bilayer. Glycoproteins present in the lipid bilayer (envelope glycoproteins) interact with receptors on the surface of various host cells and allow the retroviruses to enter the host cell. Once in the cell, the retroviruses reverse transcribe the RNA of the viral genome into a double-stranded DNA (a proviral intermediate), and incorporate the deoxyribonucleic acid (DNA) into the cellular genome as a provirus. Gene products from the integrated foreign DNA may then be produced so that progeny viral particles may be assembled. As retroviruses can be modified to carry exogenous nucleotide sequences of interest, such recombinant retroviruses have a variety of uses. For example, such recombinant retroviruses are important in introducing desired exogenous

sequences into a cell, so that relatively high levels of the protein encoded by the sequences may be produced. However, use of such recombinant retroviruses has several drawbacks.

For example, retroviruses do not have a broad host range. Efforts at increasing the host range of retroviruses have included substituting the envelope glycoproteins of the virus with that of a different virus, thus forming a pseudotyped retrovirus. The pseudotyped retrovirus advantageously has the host range of the different virus. However, some retroviruses have been pseudotyped with viral glycoproteins that are toxic to cells, so the cells can only produce the virus for a limited time. Furthermore, in many cases, the pseudotyped retroviruses can not be stably produced and may not be produced at a high titer.

There is therefore a need for pseudotyped retroviruses of broad host range, and cell lines capable of producing such pseudotyped retroviruses. The present invention addresses this need.

SUMMARY OF THE INVENTION

It has been discovered that cells may be constructed to produce inventive retroviruses pseudotyped with viral glycoproteins, wherein the retroviruses have a broad host range. Accordingly, one aspect of the invention provides eukaryotic cells that include a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one preferred embodiment, the fourth nucleotide sequence encodes at least two different viral glycoproteins, preferably togaviral glycoproteins, such as, for example, alphaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a filoviral glycoprotein, such as, for example, a Marburg virus or Ebola virus glycoprotein. In a preferred form of the invention, the cells stably produce inventive pseudotyped retroviruses.

A second aspect of the invention provides methods of forming the above-described eukaryotic cells. The method includes transfecting a eukaryotic cell with a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one preferred embodiment, the fourth nucleotide sequence encodes at least two different viral glycoproteins, preferably togaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a filoviral glycoprotein, such as a Marburg virus glycoprotein. In preferred forms of the invention, the first, second, third and fourth nucleotide sequences are chromosomally-integrated, wherein the cell stably produces inventive pseudotyped retroviruses.

A third aspect of the invention provides inventive pseudotyped retroviruses, including a retroviral capsid, a lipid bilayer surrounding the retroviral capsid and at least one viral glycoprotein disposed in the lipid

bilayer. In inventive pseudotyped retroviruses, at least two different viral glycoproteins are disposed in the lipid bilayer, and in preferred embodiments, the viral glycoproteins are togaviral glycoproteins. In an alternative embodiment, the viral glycoprotein is a filoviral glycoprotein, preferably a Marburg virus glycoprotein.

In yet a fourth aspect of the present invention, methods of introducing nucleotide sequences into a cell are provided, and include transducing a cell permissive for viral entry with a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer surrounding the retroviral capsid, at least one viral glycoprotein disposed in the lipid bilayer and a desired ribonucleotide sequence. In one preferred form of the invention, the cells are permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer, such as a togavirus wherein the viral glycoproteins are togaviral glycoproteins. In alternative embodiments, the viral glycoprotein is a filoviral glycoprotein, preferably a Marburg virus glycoprotein.

A fifth aspect of the invention provides methods of screening agents effective in blocking viral entry into a cell. In one mode of practicing the invention, the method includes treating a pseudotyped retrovirus with the agent, treating a cell permissive for viral entry with the treated pseudotyped retrovirus and identifying eukaryotic cells having the desired marker. In one embodiment, the pseudotyped retrovirus has a retroviral capsid, a lipid bilayer surrounding the capsid, at least two different viral glycoproteins disposed in its lipid bilayer, such as togaviral glycoproteins wherein the cell is permissive for togaviral entry, and a nucleotide sequence encoding a desired marker. In alternative embodiments, a method is provided for screening agents effective in blocking filoviral entry, preferably Marburg virus entry, into a cell. Pseudotyped retroviruses having Marburg virus glycoprotein disposed in their lipid bilayer are preferred as are cells permissive for Marburg virus entry.

In yet another embodiment of a method of screening agents effective in blocking viral entry into a cell, the method includes treating a cell permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer with said agent, contacting the treated cell with a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer that surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer, such as togaviral glycoproteins wherein the cell is permissive for togaviral entry, and a nucleotide sequence encoding a desired marker, and identifying cells having the marker. In alternative embodiments, a method is provided for screening agents effective in blocking filoviral entry, preferably Marburg virus entry, into a cell. Pseudotyped retroviruses having Marburg virus glycoprotein disposed in their lipid bilayer are preferred as are cells permissive for Marburg virus entry.

In a sixth aspect of the present invention, kits for forming inventive pseudotyped retroviruses are provided. The kits include a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one embodiment, the fourth nucleotide sequence encodes at least two viral glycoproteins, such as togaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a Marburg virus glycoprotein.

One object of the invention is to provide a eukaryotic cell including a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein, such as a Marburg virus glycoprotein, preferably at least two viral glycoproteins, such as togaviral glycoproteins and especially alphaviral glycoproteins.

Another object is to provide a eukaryotic cell that includes a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein, such as a Marburg virus glycoprotein, preferably at least two viral glycoproteins, such as togaviral glycoproteins and especially alphaviral glycoproteins, wherein the cell stably produces the inventive pseudotyped retroviruses.

Another object is to provide a method of making the inventive cells described above, as well as the pseudotyped retroviruses so produced.

Other objects are to provide a method of screening agents effective in blocking either filoviral entry into a cell or entry of viruses having more than one viral glycoprotein in their lipid bilayer, such as togaviruses, and methods of introducing desired nucleotide sequences into a cell.

Yet other objects of the invention are to provide kits for forming inventive pseudotyped retroviruses.

These and other objects and advantages of the present invention will be apparent from the descriptions herein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows a Western blot of proteins derived from lysates of stable cell line SafeRRnlslacZ, or precursor gpnlslacZ cells, as further described in Example 4.

FIG. 2 depicts Giemsa solution-stained SafeRR-nlslacZ cells (Panel A, FIG. 2A) and ΦNX cells (Panel B, FIG. 2B) after being incubated at room temperature for one hour with pH 5.5 fusion buffer and grown in D-MEM FBS/PS culture medium for four hours as described in Example 5. Panel C (FIG. 2C) depicts Giemsa solution-stained SafeRR-nlslacZ cells treated in a similar manner with the exception that they were exposed to pH 7 fusion buffer instead of pH 5.5 fusion buffer.

FIG. 3 depicts graphs showing the effects of lysosomotropic agents on transduction of the indicated retroviruses. Left panel, A, FIG. 3A, shows the effect of ammonium chloride and right panel, B, FIG. 3B, shows the effect of chloroquine. RRV, pseudotyped virus obtained from supernatants of SafeRR-nlslacZ cells; Mo-MuLV, wild type Moloney murine leukemia virus expressing the env glycoprotein; VSV;Moloney murine leukemia virus pseudotyped with vesicular stomatitis viral glycoprotein G.

FIG. 4 shows fluorescence profiles of NIH 3T3 cells transduced with supernatant medium from ΦNX cells (top panel, A, FIG. 4A) or Safe-Ebola-GFP cells (bottom panel, B, FIG. 4B) according to the procedure outlined in Example 9.

FIG. 5 depicts syncytia formation by packaging cells expressing Ebola glycoprotein. The cells were treated according to the protocol in Example 10. Top panel, A, (FIG. 5A) SafeEbola-GFP cells; Bottom panel, B, FIG. 5B, ΦNX cells.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to preferred embodiments and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations and further modifications of the invention, and such further applications of the principles of the invention as illustrated herein, being contemplated as would normally occur to one skilled in the art to which the invention relates.

The present invention relates to eukaryotic cells that stably produce pseudotyped retroviruses and methods for their production, pseudotyped retroviruses, methods of introducing nucleotide sequences into a target cell, methods of screening agents effective in blocking viral entry into cells and kits for forming inventive pseudotyped retroviruses.

It has been discovered that eukaryotic cells may be constructed that either transiently or stably produce pseudotyped retroviruses having at least two different viral glycoproteins disposed in their lipid bilayer, such as togaviral glycoproteins. It has further been discovered that eukaryotic cells may be constructed that stably produce pseudotyped retroviruses having filoviral glycoproteins disposed in their lipid bilayer. The pseudotyped retroviruses of the present invention are advantageous in transducing cells of interest, are not toxic to the cells, have a broad host range and do not allow for pseudotransduction (i.e., introduction of proteins and/or genetic material without stable transmission of genetic material). Moreover, the present disclosure is the first report of a pseudotyped retrovirus having two different viral glycoproteins, with different membrane spanning domains, disposed in its lipid bilayer.

Accordingly, one aspect of the invention provides inventive eukaryotic cells having nucleotide sequences encoding retroviral Gag polypeptide, retroviral Pro polypeptide, retroviral Pol polypeptide and at

least one viral glycoprotein, such as a filoviral glycoprotein, or at least two viral glycoproteins, such as togaviral glycoproteins. In a preferred embodiment, nucleotide sequences encoding the polypeptides described are chromosomally-integrated and thus stably produce inventive pseudotyped retroviruses. A second aspect of the invention provides methods of forming cells that produce inventive pseudotyped retroviruses. A third aspect of the invention provides the inventive pseudotyped retroviruses, preferably those that include at least two different viral glycoproteins disposed in their lipid bilayer, including togaviral glycoproteins, and further preferably those that include a desired nucleotide sequence in their genome. Other aspects of the invention provide inventive methods of introducing a nucleotide sequence into a desired cell and methods of screening agents effective in blocking viral entry into a target cell, preferably blocking entry of a Marburg virus, or a virus having more than one viral glycoprotein in its lipid bilayer such as a togavirus, wherein all of the methods utilize the inventive pseudotyped retroviruses and cells described above, and kits for producing inventive pseudotyped retroviruses.

As discussed above, one aspect of the invention provides eukaryotic cells, forming inventive eukaryotic cell lines, having nucleotide sequences encoding retroviral Gag polypeptide, retroviral Pro polypeptide, retroviral Pol polypeptide and at least one viral glycoprotein, such as a filoviral glycoprotein, or at least two different viral glycoproteins, typically from the same virus, such as togaviral glycoproteins. The term "eukaryotic cell line" as used herein is intended to refer to eukaryotic cells that are grown *in vitro*. The term "nucleotide sequence", as used herein, is intended to refer to a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, and derivatives thereof. The terms "encoding" and "coding" refer to the process by which a nucleotide sequence, through the mechanisms of transcription and translation, provides the information to a

cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce a polypeptide.

In forming a cell that produces an inventive pseudotyped retrovirus, a wide variety of cells may be selected. Eukaryotic cells are preferred, whereas mammalian cells are more preferred, and include human, simian canine, feline, equine and rodent cells. Human cells are most preferred. It is further preferred that the cell be able to reproduce indefinitely, and is therefore immortal. Examples of cells that may be advantageously used in the present invention include NIH 3T3 cells, COS cells, Madin-Darby canine kidney cells and human embryonic 293T cells. However, highly transfectable cells, such as human embryonic kidney 293T cells, are preferred. By "highly transfectable" it is meant that at least about 50%, more preferably at least about 70% and most preferably at least about 80% of the cells can express the genes of the introduced DNA.

The retroviral *gag*, *pro* and *pol* nucleotide sequences, and other retroviral nucleotide sequences for forming the specified pseudotyped retroviruses, may be obtained from a wide variety of genera in the family Retroviridae, including, for example, Oncoviruses, including Oncovirus A, B, C and D, lentiviruses and spumavirus F. Such sequences are preferably obtained from the Moloney murine leukemia virus (MMLV; in the genus Oncovirus C). Such sequences are well known in the art. For example, nucleotide sequences encoding MMLV gag, pro and pol may be found in Bereven et al., *Cell* (1981) 27:97-108. Most preferably, such sequences are obtained from lentiviruses. Unlike most retroviruses, lentiviruses have the capacity to integrate the genetic material they carry into the chromosomes of non-dividing cells as well as dividing cells. Therefore, lentiviral nucleotide sequences encoding proteins that allow for chromosomal integration of virally transported nucleic acid in non-dividing cells are advantageously employed, as the host range of the pseudotyped retroviruses will be broadened.

The above-described retroviruses are readily publicly available from the American Type Culture Collection (ATCC) and the desired nucleotide sequences may be obtained from these retroviruses by methods known to the skilled artisan. For example, the nucleotide sequences may be obtained by recombinant DNA technology. Briefly, viral DNA libraries may be constructed and the nucleotide sequences may be obtained by standard nucleic acid hybridization or polymerase chain reaction (PCR) procedures, using appropriate probes or primers. Alternatively, supernatant medium from cells infected with the respective virus can be isolated and the desired retroviral nucleotide sequences may be amplified by PCR. Such vectors may also be constructed by other methods known to the art.

It is preferred that the *gag*, *pro* and *pol* nucleotide sequences are contiguous to each other as found in native retroviral genomes, such as in the order 5'-gag-pro-pol-3'. It is further preferred that these retroviral nucleotide sequences are chromosomally-integrated into the cellular genome. Furthermore, the gag-pro-pol nucleotide sequences are operably linked at the 5' end of the *gag* nucleotide sequence to a promoter sequence, so that transcription of the sequences may be achieved.

A nucleic acid sequence is "operably linked" to another nucleic acid sequence, such as a promoter sequence, when it is placed in a specific functional relationship with the other nucleic acid sequence. The functional relationship between a promoter and a desired nucleic acid typically involves the nucleic acid and the promoter sequences being contiguous such that transcription of the nucleic acid sequence will be facilitated. Two nucleic acid sequences are further said to be operably linked if the nature of the linkage between the two sequences does not (1) result in the introduction of a frame-shift-mutation; (2) interfere with the ability of the promoter region sequence to direct the transcription of the desired nucleotide sequence, or (3) interfere with the ability of the desired nucleotide sequence to be transcribed by the promoter sequence region.

Typically, the promoter element is generally upstream (i.e., at the 5' end) of the nucleic acid coding sequence.

A wide variety of promoters are known in the art, including cell-specific promoters, inducible promoters, and constitutive promoters. The promoters may further be selected such that they require activation by activating elements known in the art, so that production of the protein encoded by the specified nucleic acid sequence may be regulated as desired. It is well within the purview of a person skilled in the art to select and use an appropriate promoter in accordance with the present invention. For example, the promoters that may be advantageously present in the cell, 5' to the gag-pro-pol sequences, include rat actin promoter and the MMLV promoter. Furthermore, the cytomegalovirus promoter has been found to be an excellent promoter in the inventive system.

Other regulatory elements, such as enhancer sequences, which cooperate with the promoter and transcriptional start site to achieve transcription of the nucleic acid insert coding sequence, may also be present in the cell 5' to the nucleotide sequences that encode retroviral proteins. By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity in a cell, such as a bacterial or eukaryotic host cell.

A wide variety of viral glycoproteins may be advantageously present in the inventive cells of the present invention, especially viral glycoproteins necessary for attachment of the virus to a target cell and penetration of the virus into the cytoplasm of the cell, as well as viral glycoproteins necessary for maturation of the glycoproteins necessary for attachment and penetration of the virus. For example, the cells described above may include nucleotide sequences encoding at least two different viral glycoproteins. Examples of such viruses include viruses in the families Togaviridae (e.g., in the genus *Alphavirus* or *Rubivirus*), Flaviviridae (e.g., *Flavivirus*, *Pestivirus* and *Hepatitic C*), Paramyxoviridae (e.g., Morbillivirus), and Bunyaviridae (e.g., Hantavirus). Such nucleotide sequences are well

known to the art. In one embodiment, the cells may include, instead of the viral nucleotide sequences encoding at least two different viral glycoproteins, nucleotide sequences encoding filoviral glycoproteins. Examples of such viruses include Ebola virus (including Ebola Zaire, Ebola Reston and Ebola Sudan sequences which are chromosomally-integrated), and Marburg virus. These nucleotide sequences may be obtained by methods known in the art as recited in example 2. For example, nucleotide sequences encoding particular glycoproteins may be isolated and cloned into plasmids by standard techniques, and the nucleotide sequence may then be amplified by PCR using the appropriate primers.

In one form of the present invention, the cells include nucleotide sequences encoding glycoproteins from an alphavirus. In a most preferred embodiment, the cells include nucleotide sequences encoding glycoproteins from the viral species Ross River (depicted in SEQ ID 1). The viral transmembrane glycoprotein complex that is responsible for the binding of the alphavirus to the surface of a susceptible cell and for the fusion of the viral and cellular membranes that occurs during the process of viral entry includes a trimer of a heterodimer of two transmembrane proteins, which are denoted E_1 and E_2 and which are encoded by an E_3-E_2-6K-E_1 glycoprotein coding region (E_3 and 6K refer to viral proteins involved in maturation of E_1 and E_2 as known in the art) on the alphaviral genome. The E_2-E_1 coding region includes an E_3 glycoprotein coding region as well as the 6K protein coding region. Such nucleotide sequences may be obtained by methods known to the skilled artisan as discussed for the *gag*, *pro* and *pol* nucleotide sequences above. For example, the E_2-E_1 coding region may be obtained as discussed in Kuhn et al. (1991) *Virology* 182:430-441. The E_2-E_1 glycoprotein coding region is also operably linked to a promoter sequence, such as described above, at its 5' end.

The eukaryotic cells described above, that include nucleotide sequences encoding togaviral glycoproteins, advantageously produce retroviruses pseudotyped with togaviral glycoproteins at a titer of at least

about 1 x 10^3 transforming units (TU)/ml of cell culture supernatant medium. The cells more preferably produce such retroviruses at a titer of at least about 1 x 10^5 TU/ml of supernatant and most preferably at a titer of at least about 1 x 10^6 TU/ml of supernatant.

It is expected that other viruses not specifically mentioned herein having at least two different glycoproteins of similar structure to the glycoproteins in the viral families denoted above may be advantageously used in the present invention.

In another embodiment, the cells include nucleotide sequences encoding glycoproteins from a filovirus. Such filoviruses also exhibit a broad host range. A wide variety of nucleotide sequences that encode filoviral glycoproteins may be used to produce the inventive cells of the present invention. For example, nucleotide sequences encoding glycoproteins from the Marburg and Ebola virus (in the family Filoviridae and, including, for example, Ebola-Zaire and Ebola-Reston) may be introduced into the cells described above to produce a pseudotyped retrovirus. SEQ ID 2 shows the Ebola Zaire glycoprotein-encoding sequence and SEQ ID 3 shows the Marburg virus glycoprotein-encoding sequence. The nucleotide sequences encoding the filoviral glycoproteins may be obtained as described in Sanchez et al. (1993) *Virus Res.* 29 (3):215-240 and Will et al., (1993) *J. Virol.* 67:1203-1210. Moreover, such sequences may be obtained by other methods known to those skilled in the art, as described above for the togaviruses.

Eukaryotic cells described above that include the filoviral nucleotide sequences advantageously produce retroviruses pseudotyped with a filoviral glycoprotein at a titer of at least about 4.5 x 10^4 TU/ml of supernatant. The cells more preferably produce such retroviruses at a titer of at least about 1 x 10^6 TU/ml of supernatant and most preferably at a titer of at least about 1x 10^7 TU/ml of supernatant.

It is expected that other viruses not specifically mentioned above and having glycoproteins of similar structure to the filoviral glycoproteins may be advantageously used in the present invention.

The cells may transiently produce the retrovirus pseudotyped with at least two different viral glycoproteins, such as togaviral glycoproteins, or with a filoviral glycoprotein, but preferably stably produce such retroviruses. In one preferred form of the present invention, the nucleotide sequences encoding either the filoviral glycoproteins or encoding at least two different viral glycoproteins (such as togaviral glycoproteins) in the eukaryotic cells are chromosomally-integrated, so that the cell stably produces the pseudotyped retrovirus. By "stably produce", it is meant that the cells will produce pseudotyped retrovirus indefinitely (i.e., during the life span of the cell). Conversely, by transient production, it is meant that the cells will produce pseudotyped retrovirus for a period of at least about 24 hours, more preferably at least about 48 hours, and most preferably at least about 72 hours.

In a further preferred form of the present invention, the eukaryotic cells described above may include another nucleotide sequence that encodes a desired protein so that they may produce pseudotyped retroviruses having an RNA genome including such desired nucleotide sequences. The protein can be such that it provides a beneficial or therapeutic effect if introduced into an animal. For example, a gene may encode a protein that is needed by an animal, either because the protein is no longer produced, is produced in insufficient quantities to be effective in performing its function, or is mutated such that it either no longer functions or is only partially active for its intended function. The nucleotide sequence may be introduced into the cellular genome in a variety of ways known to the skilled artisan. For example, defective retroviruses (i.e., those which do not have the capability to produce all of the viral proteins necessary for production of a retrovirus having the ability to infect a cell and produce progeny viruses) may be constructed to include such a sequence in their

RNA genome and can then transduce a cell. Alternatively, and as described above, plasmid vectors may be used to introduce the nucleotide sequence, preferably DNA, encoding the desired protein. In either case, the vector typically includes nucleotide sequences necessary for production of the pseudotyped retrovirus. For example, the RNA sequence in the viral genome is flanked on the 5' end by a splice acceptor site and a splice donor site followed by a sequence necessary for packing of the viral genome (such as a psi sequence) and a long terminal repeat (LTR), all as known in the art. The 3' end of the RNA sequence may be flanked on its 3' end with a polypurine tract followed by another LTR, further as known to the skilled artisan. The vectors may include other nucleotide sequences known to the art that are necessary for transduction.

In one preferred form, the desired protein may be one that allows entry of the virus into a cell to be detected. For example, a visually detectable component, or marker, such as one that emits visible wavelengths of light, or that may be reacted with a substrate to produce color of specified wavelengths. For example, such nucleotide sequences include the nucleotide sequence encoding the *Aequorea victoria* green fluorescent protein [GFP; nucleotide sequences listed in Prasher et al., (1992) *Gene* 111:229] and the LacZ gene (produces β-galactosidase), both of which are well known in the art and may be obtained commercially.

A second aspect of the invention provides methods of forming eukaryotic cells for producing pseudotyped retroviruses. The method includes introducing into the cells described above the nucleotide sequences described above, i.e., those encoding the retroviral Gag, Pro and Pol polypeptides, and those encoding either a filoviral glycoprotein or at least two different viral glycoproteins, such as togaviral glycoproteins, into the cell.

The nucleotide sequences may be introduced into the desired cell utilizing a variety of vectors known to the skilled artisan. For example, plasmid vectors, cosmid vectors, and other viral vectors, such as retroviral

vectors, may be used. It is preferred that the nucleotide sequences encoding the Gag, Pro and Pol polypeptides are on a separate vector than the nucleotide sequences encoding the viral glycoproteins.

In one mode of practicing the invention, plasmid vectors are advantageously used to introduce, or transfect, the nucleotide sequences into the selected cell. A wide variety of plasmid vectors may be used, including pTRE, pCMV-Script and pcDNA3, although pcDNA3 is a preferred vector. The *gag, pro* and *pol* nucleotide sequences are preferably on the same plasmid, and, as discussed above, are preferably contiguous to each other. However, the skilled artisan is aware that other spatial configurations of the nucleotide sequences may be utilized when constructing the plasmids. The vector also preferably includes a promoter 5' to, or upstream from, the *gag* nucleotide sequence. The vectors may further include other regulatory elements, such as enhancer sequences, as discussed above.

The nucleotide sequences encoding the viral glycoproteins are preferably on a separate plasmid, or other vector, than the *gag, pro* and *pol* nucleotide sequences. The viral glycoprotein encoding sequences, such as the sequences encoding either the filoviral glycoproteins or those encoding at least two different viral glycoproteins (such as togaviral glycoproteins) are also preferably operably linked to a promoter sequence described above. It is also understood that the nucleotide sequences encoding at least two different viral glycoproteins may be arranged on a vector such that the nucleotide sequences encoding one of the glycoproteins are present on one vector and the sequences encoding the other glycoprotein are present on a different vector. It is preferred, however, that such sequences are on the same vector, and preferably contiguous to each other so they will be transcribed utilizing the same promoter. In one preferred form of the invention, the promoter sequence is a cytomegalovirus promoter sequence. Plasmids, or other vectors carrying the nucleotide sequences encoding the viral glycoproteins, may

also include other regulatory elements, such as enhancers, as described above.

The vectors may be introduced into the cells in a variety of ways known to the skilled artisan, for example, discussed in *Current Protocols in Molecular Biology*, John Wiley and Sons, edited by Ausubel et al. (1988) and Maniatis, et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory (1989). For example, vectors may be transfected into a cell by a calcium phosphate precipitation method. Other methods for introduction of the vectors include, for example, electroporation and lipofection.

The nucleotide sequences may be introduced into the cells by a transient transfection procedure such that the proteins encoded by the respective sequences will be produced in a transient fashion as described above. By introducing the MMLV gene sequences and the E_2-E_1 coding region from the Ross River virus (RRV) described above into a cell, we have determined that the cell lines produce pseudotyped retrovirus for a period of about 48 hours. However, it is preferred that the sequences are stably introduced. That is, it is preferred the nucleotide sequences become integrated into chromosomes of the cells into which they are introduced. In this way, the cells will stably produce pseudotyped retrovirus for a longer period of time compared to the transient expression. As used herein, a "stable cell line" or "stable cell" is defined as one that has chromosomally-integrated the nucleotide sequences described above and can produce pseudotyped retrovirus indefinitely (i.e., for the life span of the cell).

Furthermore, in order to form such stable cells, it is necessary to use selectable markers to screen for cells which have chromosomally-integrated the introduced DNA. Accordingly, the plasmid vectors, or other vectors, into which the respective nucleotide sequences are cloned may include such selectable markers.

A wide variety of selectable markers may be used. Typical selectable markers allow growth of only those cells which have been

transfected or transduced and thereby stably produce a desired protein. Examples of selectable markers that may be used include antibiotic resistance genes, including the neomycin gene, the hygromycin phosphotransferase gene and the bleomycin resistance gene which confer resistance to G418, hygromycin and zeocin, respectively. Other selectable markers include, for example, mutant mouse dihydrofolate reductase gene (confers resistance to methotrexate), and the bacterial gpt gene (selects for cells that can grow in a medium containing mycophenolic acid, xanthin and aminopterin). These selectable markers are discussed in *Retroviruses*, Cold Spring Harbor Laboratory Press, p. 444, edited by Coffin, J.M, Hughes, S.H. and Varmus, H.E. (1997).

In many cases, one may wish to quickly visually detect those cells which have taken up a vector and that produce a specified protein from the vector. Visually detectable components, or markers, include the *Aequorea victoria* green fluorescent protein as discussed above. When forming a cell that includes a visually detectable component, or marker, the nucleotide sequences encoding the marker may also be introduced into the cell as described above. For example, the nucleotide sequence encoding the green fluorescent protein may be placed in a recombinant MMLV genome or in a plasmid (to form plasmid MFG.S-GFP) by methods known to the art. For example, plasmid MFG.S-GFP may be formed by including in plasmid MFG [produced by methods known in the art and as exemplified by Ory et al., *PNAS USA*, 93:11400-11406 (1996)] the nucleotide sequence encoding the green fluorescent protein, surrounded by the nucleotide sequences described above, such as LTRs and the psi sequence. Cells that have taken up the vector and express the nucleotide sequences encoding a protein may be identified and separated from cells that do not express the sequences by a fluorescensce-activated cell sorting procedure as known in the art. A visually detectable marker may also be formed from reaction of β-galactosidase (produced by the LacZ gene) with a substrate, such as X-gal.

Moreover, when growing cells that produce inventive pseudotyped retroviruses, the cells should be grown to no more than about 50% confluency, more preferably no more than about 25% confluency, and the pH of the culture medium should be maintained at about 7 by the frequent changing of culture medium. These conditions are conducive for production of cells that stably produce the pseudotyped retroviruses and should be strictly followed.

In a third aspect of the present invention, pseudotyped retroviruses that include viral glycoproteins (as discussed above) disposed in their lipid bilayer are provided. In one embodiment, at least two different viral glycoproteins are present in the lipid bilayer, such as togaviral glycoproteins. In alternative embodiments the glycoprotein is a filoviral glycoprotein.

In one embodiment, such pseudotyped retroviruses include a core RNA genome that is surrounded by, or enclosed within, a viral capsid. The genome preferably includes a nucleotide sequence encoding a protein selected to be subsequently produced by a cell. The genome further includes other nucleotide sequences for formation of the pseudotyped retrovirus, such as 5' and 3' LTR sequences that are operably linked to the nucleotide sequence encoding the desired protein as described above. Reverse transcriptase and integrase are also enclosed within the capsid, which gives the retrovirus the ability to incorporate a gene encoding a desired protein into a genome of a cell after the retrovirus contacts, or is incubated with, the cell. For example, the pseudotyped retrovirus may be used to incorporate a gene encoding an enzyme in a host cell that is incapable of producing the enzyme, or produces a non-functional enzyme as discussed above. Other sequences known to the art that are useful for transducing genes may also be present in the RNA genome.

The pseudotyped retrovirus may include other proteins, in addition to integrase, that aid its stable integration into the chromosomes of a target

cell. For example, with respect to a lentivirus, the pseudotyped retrovirus may include proteins such as vpr, vif and vpu.

In yet other preferred embodiments, the pseudotyped retrovirus may include a nucleotide sequence encoding a visually detectable component, or marker, such as *Aequorea victoria* green fluorescent protein as discussed above. Such a retrovirus may be advantageously used in a method of determining viral entry into a cell discussed above. Moreover, such a virus is advantageously used in the methods of the present invention to ensure that the pseudotyped retroviruses that are formed are replication incompetent (i.e., do not have all the sequences necessary in their viral genome to produce progeny retroviruses). For example, supernatant isolated from cells transduced by the vectors and contacted with a test cell should not result in localization of the fluorescent protein in the test cell.

In a fourth aspect of the present invention, methods of introducing nucleotide sequences into a cell are provided. In one embodiment, the method includes contacting, or transducing, a cell permissive for either filoviral entry, or entry of a virus having at least two different viral glycoproteins in its lipid bilayer such as a togavirus, with a retrovirus that has been pseudotyped with a filoviral glycoprotein or at least two different viral glycoproteins, such as togaviral glycoproteins, as described above that includes the desired nucleotide sequence in its genome. When the nucleotide sequences encode a desired protein, the cell is selected so that it also preferably allows expression of the selected nucleotide sequence. The level of transduction may be obtained by assaying methods known to the skilled artisan, and include assaying for the protein of interest encoded by the introduced nucleotide sequences or assaying for the presence of the nucleotide sequences. Viruses having at least two different viral glycoproteins in their lipid bilayer have a broad host range. For example, as togaviruses are pantropic (i.e., can invade, or infect, many different cell types with no special affinity for any particular cell type), a wide variety of

permissive cell types well known to the art may be chosen for use in the method, including for example, skin cells, muscle cells, fibroblasts, fat cells and central nervous system cells.

Other viruses having at least two viral glycoproteins in their lipid bilayer include those previously described above. Cells permissive for these viruses are well known to the skilled artisan. Similarly, as filoviruses infect a broad range of cells, a wide variety of cells known to the art that are permissive for filovirus entry may also be selected, including, for example, kidney cells, liver cells, muscle cells and fibroblasts.

In a fifth aspect of the present invention, methods of screening agents effective in blocking viral entry into a cell are provided. The methods allow for direct screening as the viral entry step can be detected in the method. If such agents were tested with a wild type virus, for example, multiple rounds of replication may occur and steps other than viral entry may thus be affected (e.g., such as replication of RNA, production of proteins, etc.). In such a case, one would not know if the agent affects the entry step or some other, indirect step. Thus, the present method allows for direct quantitation of viral entry as compared to remote quantitation.

In one embodiment of the methods of the present invention, a method includes (a) treating a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer that surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer and a nucleotide sequence encoding a marker, preferably a visually detectable marker (or one that is capable of visual detection as described above) that is enclosed within the retroviral capsid, with an agent effective in blocking entry into a cell of the virus having at least two different viral glycoproteins in its lipid bilayer to form a treated pseudotyped retrovirus; (b) treating a cell permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer with the treated pseudotyped retrovirus; and (c) identifying cells having the desired marker. In one embodiment, the retrovirus may have togaviral glycoproteins disposed in its lipid bilayer, and

the cells are permissive for togaviral entry. In alternative embodiments, the retrovirus may have a filoviral glycoprotein, such as a Marburg virus glycoprotein, disposed in its lipid bilayer, wherein the cells that are treated are permissive for Marburg virus entry.

Cells that are advantageously used in a method of screening agents effective in blocking viral entry into a cell are those that are permissive for entry of the specific virus, and will therefore depend on the virus used. Cells permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer are the same as recited in the method of introducing nucleotide sequences into a cell as discussed above. Similarly, cells permissive for Marburg virus entry include those described above used in the method of introducing nucleotide sequences into a cell. If it is not known whether a cell is permissive for viral entry, this can readily be determined by the skilled artisan using routine procedures. One way of determining whether a cell is permissive for viral entry is to transduce the cell with a pseudotyped retrovirus of the present method encoding a marker, and cells that have the marker may be identified by methods known to the art. The marker may be a visually detectable marker, such as the green fluorescent protein or β-galactosidase (i.e., one that gives rise to a visually detectable marker) described above. The selected cell should also allow for expression of the gene products encoded and carried on the viral genome

A wide variety of agents may advantageously be screened in the present invention, including, immunological agents such as monoclonal and/or polyclonal antibodies. For example, monoclonal antibodies or polyclonal antisera against E_2, or other viral glycoproteins, may advantageously be used. Various pharmacological agents may also be screened in the present method in the same way, and include proteins, peptides or various chemical agents.

In one preferred method, the vector, in (a) above, is treated, or incubated with, the agent for a time period sufficient for interaction of the

agent with the viral glycoprotein. Although this time period may vary depending on the nature of the agent and the viral glycoprotein, agents effective in blocking viral entry tend to effectively interact with the glycoprotein in a period of about 10 to about 60 minutes.

In (b), the cell is incubated, or contacted, with the treated pseudotyped retrovirus for a time period sufficient for viral entry. This time period may vary, depending on the specific cell type chosen and the specific viral glycoprotein present in the lipid bilayer of the pseudotyped retrovirus as the skilled artisan knows. However, the time period can typically range from about 1 to about 6 hours, but is typically about 1 to about 2 hours.

Cells having the desired marker may be identified in (c) by observing the presence of the marker. Any of the visually detectable markers previously described above may be utilized in the method. However, a preferred marker is the *Aequorea victoria* green fluorescent protein. Cells into which this marker has been introduced may be identified and separated from cells without the marker (cells not transduced by the retrovirus) by fluorescence-activated cell sorting as described above.

Furthermore, yet another embodiment of a method of screening agents effective in blocking viral entry into a cell includes (1) treating a cell permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer with the agent to form a treated cell; (2) contacting the treated cell with a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer that surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer and a nucleotide sequence encoding a marker, preferably a visually detectable marker (or one that is capable of visual detection as described above), that is enclosed within the retroviral capsid; and (3) identifying cells having the desired marker. As above, the retrovirus may have togaviral glycoproteins disposed in its lipid bilayer, and the cells are permissive for togaviral entry. In alternative embodiments, the retrovirus may have a filoviral glycoprotein,

such as a Marburg virus glycoprotein, disposed in its lipid bilayer, wherein the cells that are treated are permissive for Marburg virus entry. The cells and agents advantageously used in this embodiment are the same as described in the previous embodiment.

In this alternative embodiment, the cells in (1) above are treated, or incubated with, the agent for a time period sufficient for interaction of the agent with the cell to form a treated cell. Although this time period may vary depending on the nature of the agent and the cell, agents effective in blocking viral entry tend to effectively interact with the cell in a period of about 1 hour.

In (2), the treated cell is incubated, or contacted, with the pseudotyped retrovirus for a time period sufficient for viral entry. The time period may vary, depending on the specific cell type chosen and the specific viral glycoprotein in the lipid bilayer of the pseudotyped retrovirus as the skilled artisan knows. However, the time period ranges from about about 1 to about 6 hours, but is typically about 1 to about 2 hours.

Cells having the desired marker may be identified in (3) by the same method as described in (c) of the previous embodiment.

In a sixth aspect of the present invention, kits for forming inventive pseudotyped retroviruses are provided. The kits include a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one embodiment of the invention, the fourth nucleotide sequence encodes at least two different viral glycoproteins, such as togaviral glycoproteins and preferably alphaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a filoviral glycoprotein, preferably a Marburg virus glycoprotein. The sequences and methods of obtaining such sequences are discussed above. In general, the kits include sterile packaging which secures the various kit components in spaced relation from one another

sufficient to prevent breakage of the components during handling of the kit. For example, it is a common practice to utilize molded plastic articles having multiple compartments or areas for holding the kit components in spaced relation.

The inventive pseudotyped retrovirus are further useful in methods of identifying cell surface receptors that allow viral entry. In one embodiment, an inventive pseudotyped retrovirus may be employed in a method that identifies cell surface receptors for a virus having at least two different viral glycoproteins disposed in its lipid bilayer. The method includes (a) constructing a cDNA library from a first cell that is permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer; (b) transfecting a second cell with a cDNA-carrying vector wherein the second cell is non-permissive or semi-permissive for entry of a pseudotyped retrovirus that includes a retroviral capsid, a lipid bilayer wherein the lipid bilayer surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer and a nucleotide sequence encoding a desired marker wherein the nucleotide sequence is enclosed within the retroviral capsid; (c) transducing the second cell with the pseudotyped retrovirus; (d) identifying cells having the marker; and (e) identifying the cDNA insert in the transduced cell. In alternative embodiments, the cDNA library is constructed from a first cell permissive for entry of a Marburg virus and the second cell is transduced with a retrovirus pseudotyped with the Marburg virus glycoprotein.

In a preferred method, the first cell is permissive for togaviral entry, further preferably alphaviral entry, and the second cell is transduced with a retrovirus pseudotyped with togaviral glycoproteins, preferably alphaviral glycoproteins.

In (a), a cDNA library may be constructed by methods well known to the skilled artisan as described in *Current Protocols in Molecular Biology*, John Wiley and Sons, edited by Ausubel et al. (1988). For example, mRNA may be isolated from the first cell by breaking the cell membrane and

extracting and purifying the mRNA by known methods. The mRNA may be used as a template to form cDNA,which may then be cloned into various vectors as described above, such as plasmid vectors, by use of various restriction enzymes and DNA ligase as known in the art. Bacterial cells, or other similar cells, may be transfected with the expression vectors to form the cDNA library.

The first cell may be chosen from the cells permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer, such as an alphavirus, or a virus having a filoviral glycoprotein disposed in its lipid bilayer, such as a Marburg virus glycoprotein, or other filovirus glycoprotein as described above.

In (c), the second cell may be transduced with a pseudotyped retrovirus having a nucleotide sequence encoding a desired marker as described above in the embodiment described above of the method for screening agents effective in blocking viral entry into a cell and in (d), the transduced cells may be identified by methods discussed above, such as fluorescence activated cell sorting.

The second cell may be selected from non-permissive cells, preferably mammalian, known in the art. For example, in the case of the pseudotyped retrovirus that includes at least two viral glycoproteins disposed in its lipid bilayer, such as those from the Ross River virus, non-permissive cells include chicken embryo fibroblasts. One skilled in the art may also determine what other cells are non-permissive for alphaviruses, such as the Ross River virus, and the filoviruses, such as Marburg or Ebola virus, by the methods described herein as well as other methods known to the art.

The cDNA insert in the transduced eukaryotic cell may be identified and recovered by known methods, including amplifying known sequences in the cDNA-containing plasmids by PCR.

Reference will now be made to specific examples illustrating the compositions and methods above. It is to be understood that the examples

are provided to illustrate preferred embodiments and that no limitation to the scope of the invention is intended thereby.

EXAMPLE 1

Cells and Cell Culture

E86nlslacZ cells, Baby Hamster Kidney (BHK) cells, and mouse NIH3T3 fibroblasts were grown in Dulbecco's Modified Eagle Media (D-MEM, Sigma) with 10% Calf Serum (Gibco-BRL), 0.1 mg/ml streptomycin (Sigma) and 10 U/ml penicillin (Sigma)(D-MEM CS/PS). E86nlslacZ cells are NIH 3T3 cells that express MMLV capsid proteins, produced as known in the art and as described in Taylor, G.M. and Sanders, D.A. (1999) *Mol. Biol. of the Cell* (1999), in press, were constructed by stably transfecting GP+E86 cells of Markowitz et al. (1988) *J. of Virol.* 62:1120-1124 with MFG.S-nlslacZ. MFG.S-nlsLacZ is a retroviral vector encoding a nuclear localized β-galactosidase activity, produced as known in the art and as described in Ory, et al. (1996) *PNAS USA* 93:11400-11406.

Human HeLa, ΦNX cells, gpGFP and gpnlslacZ cells were grown in D-MEM FBS/PS). ΦNX packaging cells are second generation human embryonic kidney 293T cells transfected with MMLV *gag* and *pol* genes as described in Grignani et al. (1998) *Cancer Res.*, 58:14-19 and Pear et al., (1993) *PNAS USA*, 90:8392-8396. gpGFP cells are obtained by transfecting ΦNX cells with retroviral vector MFG.S-GFP-S65T, a retroviral vector encoding the *Aequorea victoria* green fluorescent protein S65T mutant as described in Taylor, G.M. and Sanders, D.A. (1999) *Mol. Biol. of the Cell* (1999), in press. gpGFP cells therefore produce envelope-deficient replication- incompetent MMLV particles carrying MFG.S-GFP-S65T. gpnlslacZ cells were developed in our laboratory by cotransfecting MFG.S-nlsLacZ and pJ6Ωpuro [constructed as described in Morgenstern and Land (1990), *Nucleic Acids Res.*, 18:1068] into ΦNX cells, growing transfected cells in D-MEM FBS/PS supplemented with 2 μg/ml puromycin (Sigma) and antibiotic-resistant colonies were isolated and screened for the

production of high-titer replication-incompetent virus resulting from transient transfection with penv1min, a vector that encodes the wild type MMLV envelope protein [as described in Taylor, G.M. and Sanders, D.A. (1999) *Mol. Biol. of the Cell* (1999), in press].

VSV-G pseudotyped retrovirus-producing 293GPGnlslacZ cells, constructed as described in Ory, et al. (1996) *PNAS USA* 93:11400-11406, were grown in D-MEM FBS/PS supplemented with 2 µg/ml puromycin and 1 µg/ml tetracycline (Sigma). As expression of the VSV-G protein in these cells is repressed by the presence of tetracycline in the medium, forty-eight hours before collection of pseudotyped virus the medium in which the 293GPGnlslacZ cells were grown was replaced with D-MEM FBS/PS.

All cells were grown at 37°C and under 5% CO_2. Moreover, the cells were grown at a density of no more than about 50% confluency and the medium was changed at intervals sufficient to maintain the pH of the medium at about 7.

EXAMPLE 2

Generation of Cell Lines Transiently Producing RRV-MMLV Pseudotyped Retrovirus

RRV Glycoprotein Expression Plasmid Construction

The region encoding the Ross River virus envelope glycoproteins was amplified from pRR64, a plasmid which contains the full-length Ross River viral genome [full-length sequence described in Faragher et al., (1988), *Virology* 163:509-526] as described in Kuhn et al. (1991), *Virology* 182:430-41, by the polymerase chain reaction using Pfu polymerase and two primers complementary to the viral genome at nucleotides 8375-8386 (5'-CGGGATCCACCATGTCTGCCGCGCT-3') and 11312-11330 (5'-CGCTCTAGATTACCGACGCATTGTTATG-3') [the amplified sequences from plasmid pRR64 are shown in SEQ ID 1, beginning at nucleotide 3, and an additional "at" sequence (nucleotides 1 and 2 of SEQ ID 1) was

added at the 5' end of the pRR64 sequence]. The amplified fragment, which contained the RRV E_3-E_2-6K-E_1 coding region, was digested with the restriction endonucleases Bam HI and Xbal and ligated into BamHI and Xbal sites of pBacPac, a Baculovirus expression vector available from Clontech. The resulting plasmid was digested with BamHI and Xbal, and the fragment containing the RRV E_3-E_2-6K-E_1 coding region was ligated into the BamHI and Xbal sites in the pcDNA3 mammalian expression vector available from Invitrogen. The resulting plasmid was designated pRRV-E_2-E_1. SEQ ID 1 also shows the amino acid sequence of the E_3-E_2-6K-E_1 polypeptide.

Transient Transfection Procedure

In preparation for transfection, 0.5 x 10^6 ΦNX cells, or gpnlslacZ cells, were washed with PBS (137 mM NaCl, 27 mM KCl, 4.3 mM Na_2HPO4, 1.47 mM K_2HPO4, pH 7.4) prior to incubation with 2 ml Opti-MEM (Gibco-BRL) for 30 minutes at 37°C in a 5% CO_2 atmosphere. 2 µg of pRRV-E_2-E_1 and 2 µg of MFG.S-GFP-S65T was incubated with 300 ml Opti-MEM and 24 ml lipofectAMINE™ (Gibco-BRL) for 30 minutes at room temperature prior to dilution with 2.4 ml Opti-MEM. The resulting mixture was incubated with the cells for seven hours at 37°C in a 5% CO_2 atmosphere. Medium was replaced with DMEM FBS/PS for a further 48-hour incubation at 37°C in a 5% CO_2 atmosphere before collection of the supernatant medium for analysis of the transduction capacity of and level of glycoprotein incorporation into viral particles. When the gpnlslacZ cells were transfected, a similar protocol was followed except that the transfected DNA consisted solely of 4 µg of pRRV-E_2-E_1.

Transduction by Recombinant Retroviruses

HeLa, BHK and NIH 3T3 cells were transduced by the following method. Supernatant medium from recombinant-virus-producing cells was filtered through a 0.45 µm filter, mixed with hexadimethrine bromide

(Sigma) (final concentration 8 µg/ml) and incubated with cells for five hours at 37°C in a 5% CO_2 atmosphere. The recombinant virus-containing medium was then replaced with D-MEM CS/PS medium. Cells transduced with MFG.S-GFP-S65T were washed 48 hours after infection with PBS, then lifted from the plate with PBS containing 1 mM EDTA. The cells were then analyzed with a Coulter XL-MCL Flow Cytometer using a 525 nm band-pass and a 488 nm air-cooled argon laser. The level of glycoprotein incorporation into viral particles was determined by Western blotting as described in Example 4.

Analysis

Cell have been constructed that produce infectious pseudotyped virus containing the glycoproteins from the Ross River virus. The titer of virus was found to be 1×10^3 TU/ml supernatant. The cells were able to produce the pseudotyped retrovirus for a period of 48 hours. As MMLV only infects mouse cells such as NIH 3T3 and the Ross River virus glycoprotein-pseudotyped retrovirus is able to infect NIH 3T3 cells, as well as BHK (hamster) and HeLa (human) cells, it has clearly been demonstrated that the host range of these retroviruses is increased by incorporation of the Ross River glycoproteins in the virus.

This example also shows that at least two different viral glycoproteins, each having a different membrane-spanning domain, can be incorporated into a retroviral particle (i.e., a retrovirus).

EXAMPLE 3

Generation of Stable Cell Lines Producing RRV-MMLV Pseudotyped R trovirus

Stable Transfection Procedure

0.5×10^6 ΦNX or gpnlslacZ cells were transfected following the protocol in Example 2, except that the DNA that was transferred was only 8 µg of pRRV-E_2-E_1 and 0.4µg of plasmid pJ6Ωpuro coding for puromycin resistance [Morgenstern and Land, *Nucleic Acids Res.* 18, 1068 (1990)] and the DNA mixture contained 48 µl lipofectAMINE™ and 600 µl Optim-MEM. Selection with medium containing 2 µg/mL puromycin began at 48 hours post-transformation. Clonal colonies of cells were isolated after two weeks of selection. The resulting cell lines derived from the ΦNX cells were designated SafeRR and the resulting cell lines derived from the gpnlslacZ cells were designated SafeRRnlslacZ.

Titer was measured by infection of NIH3T3 cells as described below. Infection occurred in the presence of 8 µg/ml polybrene and infectious supernatant was changed to media without polybrene 5 hours post-infection.

Transduction by Recombinant Retroviruses

The same protocol of Example 2 was followed, except that forty-eight hours after the infection, the cells transduced with virus bearing MFG.S-nlslacZ were fixed with 0.5% glutaraldehyde (Sigma) and then incubated with 1 mg/ml of the β -galactosidase detection reagent 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Fisher) in a staining buffer (1 mM $MgCl_2$, 50 mM $K_3Fe(CN)_6$ and 50 mM $K_4Fe(CN)_6$) for 3 hours prior to the determination of the proportion of blue cells as provided in Sanes, et al. (1986), *Embo J.*, 5:3133-3142.

Analysis

Cells that permanently produce the above pseudotyped retroviruses have been constructed. SafeRR cells were found to produce pseudotyped

retrovirus at a titer of 1 x 10^3 TU/ml supernatant. SafeRR-nlslacZ cells were found to produce pseudotyped retrovirus at a titer of about 1 x 10^5 TU/ml supernatant. SafeRR cells may be advantageous in introducing desired nucleotide sequences into a cell. Another advantage is that the expression of the Ross River virus glycoproteins are not toxic to the cells.

EXAMPLE 4

Immunodetection of Incorporation of RRV-E_2 into Pseudotyped Retrovirus Produced by SafeRR-nslacZ cells

This example demonstrates that the recombinant retrovirus contains the Ross River glycoproteins.

Supernatant medium from a 10 cm tissue-culture dish of confluent SafeRRnlslacZ cells (described in Example 3), or precursor gpnlslacZ cells (described in Example 1), was passed through a 0.45 µm filter and spun through a 30% sucrose cushion at 25K rpm for 2.5 hours in a Beckman 50.2 titanium rotor. Material collected through the centrifugation was suspended in SDS-PAGE buffer (0.05% bromophenol blue, 0.0625 M Tris-HCl pH 6.8, 1% SDS, 10% glycerol). Cell lysates were prepared by washing cells with 10 ml PBS followed by 2 ml cell lysis buffer (50 mM Tris-HCL, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100). Aliquots of total lysed material were mixed with SDS-PAGE buffer and analyzed electrophoretically. PAGE-separated proteins were transferred to nitrocellulose membranes at 44 mA for 2 hours in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were blocked with 5% powdered milk in washing solution (20 mM Tris-HCl, pH7.6, 137 mM NaCl, 0.1% Tween-20). Blocked membranes were reacted with pAbE2 (anti-Ross River E_2 rabbit polyclonal antiserum; provided by Richard Kuhn and produced by methods known to the art) at a 1:5000 dilution for two hours and goat anti-rabbit Horseradish Peroxidase (HRP)-linked secondary antibody (Chemicon, 1 mg/ml) at a 1:5000 dilution for thirty minutes.

Western blots were visualized with Enhanced Chemiluminescent Reagents (Amersham Pharmacia Biotech) by methods known in the art.

Analysis

In order to clarify that E_2-E_1 were incorporated into the MMLV particles and could be mediating the infection observed in Example 3, both virus producing cells and infectious supernatants were analyzed by SDS-PAGE and Western blotting with a polyclonal E_2 antiserum.

As seen in FIG. 1, a 50kDa and a 60kDa immunoreactive protein were present in a lysate of SafeRRnlslacZ (express RRV E_2-E_1 pseudotyped MMLV). These are appropriate masses for E_2 and unprocessed E_2-E_3. Western analysis of virus collected from infectious supernatant revealed only the fully processed 50 kDa protein.

EXAMPLE 5

Formation of Syncytia in Stable SafeRR-nlslacZ Cell Lines at Acidic pH

This example shows that SafeRR-nlslacZ cell lines are capable of forming syncytia at acidic pH, implying that entry of alphavirus into cells is dependent on the low pH environment normally found in endosomes.

SafeRR-nlslacZ or ΦNX cells, obtained as described in Examples 3 and 1, respectively, were grown to near confluence, washed with PBS and treated with fusion buffer [PBS containing 10 mM 2-(N-morpholino)ethane sulfonic acid and 10 mM HEPES adjusted to pH 5.5] for one minute. The low pH solution was replaced with D-MEM FBS/PS, then cells were incubated in a CO_2 incubator at 37°C, and the cells were stained with Giemsa solution 5 hours after treatment and photographed.

Analysis

As seen in FIG. 2A, syncytia are detectable. No syncytia were observed in the treated ΦNX cells that are shown in FIG. 2B. It is seen in

FIG. 2C that syncytia are also not detected when the SafeRR-nlslacZ cells are incubated in pH 7 fusion buffer. These results, indicating that Ross River virus glycoprotein-promoted membrane fusion is triggered by an acidic medium, are consistent with the data obtained by other laboratories that indicate the entry of alphavirus is dependent upon the low pH environment normally found in endosomes [other data discussed in Strauss and Strauss, (1994) *Microbiol. Rev*, 58:491-562].

EXAMPLE 6

Effect of Lysosomotropic Weak Bases on Infection By RRV-MMLV Pseudotyped Retrovirus

This example shows that the RRV-MMLV pseudotyped retrovirus enters cells through an endocytic pathway.

NIH 3T3 cells were pretreated for one hour with various concentrations of ammonium chloride or chloroquine in PBS as seen in FIG. 3. Medium containing 1.5 x 10^5 TU/ml of supernatant of either wild type MMLV, VSV-G pseudotyped retrovirus or Ross-River E_2-E_1 pseudotyped retrovirus (produced by SafeRRnlslacZ cells) containing various concentrations of bases (as seen in FIG. 3) as well as 8 µg/ml polybrene was incubated with the cells in a CO_2 incubator at 37°C. The virus-containing medium was replaced with D-MEM CS/PS 6 hours after infection. The cells were stained with a β-galactosidase detection reagent (X-gal) at 48 hours post infection, and blue cells were counted. The results are shown in FIG. 3.

Analysis

Ammonium chloride and chloroquine inhibit the acidification of endosomes and inhibit cellular entry of viruses that are taken up by endocytosis and that require exposure to low pH for virus-cell membrane fusion to occur as reported in Marsh and Helenius, *Adv. Virus Res.* (1989), 36:107-151. MMLV entry is known not to involve low pH-induced virus-cell

membrane fusion and infection by VSV-G pseudotyped retrovirus is known to involve low pH-induced virus-cell membrane fusion. These retroviruses therefore served as controls. The results show that chloroquine only partially affects wild type MMLV entry as seen in FIG. 3A, and that both chloroquine and ammonium chloride inhibit VSV-G pseudotyped retrovirus entry. It can therefore be concluded that the dramatic inhibition of transduction by Ross River glycoprotein-pseudotyped viruses in the presence of ammonium chloride and chloroquine is a direct effect upon entry, as all of the macromolecules required for the other necessary processes (viral uncoating, reverse transcription, integration, etc.) are identical with those contained in the relatively uninhibited MMLV-Env-bearing viruses. This example illustrates one of the advantages of the inventive pseudotype system of the present invention; the effects of an experimental manipulation on viral entry into a cell may be specifically investigated independent of any effects on other steps in replication.

EXAMPLE 7

Neutralization of MMLV Pseudotyped with RRV E_2-E_1 Coding Region

This example shows that retroviruses pseudotyped with the Ross River virus E2-E1 are inhibited from entering a cell when pre-incubated with antibodies against E2.

Supernatants from SafeRR-nlslacZ or wild type MMLVnlsLacZ (MMLV that includes RNA encoding β-galactosidase and the *env* gene proteins) producing cells were incubated with dilutions of Ross River virus monoclonal 10C9 [produced as described in Smith, (1995) *PNAS USA* 92:10648-10652] in ascites fluid or dilutions of Ross River virus polyclonal (pAbE2) antiserum (provided by Richard Kuhn and produced by methods known to the art) prior to infection of NIH3T3 cells. No significant inhibition of infectivity was observed in wild type MMLVnlsLacZ while a 60% inhibition of infectivity of RRV-MMLVnlsLacZ was observed at a 1:500

dilution of polyclonal antiserum. Inhibition was most significant with monoclonal 10C9, which binds to the cell receptor binding region on RRV E_2 (Smith et al., *Proc Natl Acad Sci USA* 92, 10648-10652 (1995)). For example, a 70% inhibition of infectivity was observed in supernatant from SafeRR-nlslacZ cells with a 1:500 dilution of ascites fluid containing monoclonal 10C9.

EXAMPLE 8

Generation of Cell Lines Transiently Producing Ebola-MMLV Pseudotyped Retrovirus Including Nucleotide Sequences Encoding GFP in its Genome

This example shows production of cell lines that transiently produce MMLV pseudotyped with Ebola-Zaire glycoprotein.

pEZGP1 was produced by cloning into the polylinker of plasmid pcDNA3 nucleotide sequences corresponding to nucleotides 6029-8253 [sequences 6029-8253, corresponding to nucleotides 132-2354 described in Genbank as Accession Number U23187, are shown in SEQ ID 2 from the Ebola Zaire virus genome, with the exception that an additional "a" has been inserted between nucleotides 1027 and 1028 in SEQ ID 2 compared to the Genbank sequence] from the complete Ebola Zaire genome [described in Sanchez, et al., (1993) *Virus Res.* 29(3):215-240] obtained by digestion of the MP1153 plasmid provided by Dr. Anthony Sanchez with Eco RI and HindIII. SEQ ID 2 also shows the amino acid sequence of the Ebola Zaire glycoprotein.

gpGFP cells were transiently transfected with pEZGP1 using lipofectAMINE™ (Gibco, BRL) and Opti-MEM media (Gibco, BRL). The gpGFP cells were plated at 5x 10^5 cells/60 mm plate 24 hours prior to transfection. The cells were washed and incubated for 30 minutes at 37°C with 2 ml of Opti-MEM media. The DNA-LipofectAMINE™-Opti-MEM mixture (4µg DNA, 24 µl lipofectAMINE™, and 300 µl Opti-MEM media)

was incubated for 30 minutes at 25°C. After the 30 minute incubations, 2.4 ml of Opti-MEM media was added to the DNA-lipofectAMINE™ mixture. The resulting solution was layered onto the gpGFP cells. Eight hours later, the transfection mixture was removed and the cells were incubated with DMEM FBS/PS for 40 hours. The supernatant medium was filtered through a 0.45 µm filter and then incubated with 1 x 10^6 NIH 3T3 cells in the presence of 8 µg/ml polybrene for 4 hours. The recombinant-virus-containing medium was then replaced with D-MEM CS/PS. Forty-eight hours later the cells were removed from the plate, suspended in 1xPBS containing 1 mM EDTA, and analyzed by flow cytometry with a Coulter XL-MCL Flow Cytometer, using a 525 nm band-pass filter and a 488 nm air-cooled argon laser.

Analysis

Cell have been constructed that produce infectious pseudotyped virus containing glycoproteins from the Ebola Zaire virus. The titer of virus was found to be 4.5 x 10^4 TU/ml of supernatant. The cells were able to produce the pseudotyped retrovirus for a period of about 24 hours.

EXAMPLE 9
Generation of Stable Cell Lines Producing Ebola-MMLV Pseudotyped Retrovirus

gpGFP cells were stably transfected with pEZGP1. gpGFP cells were plated at 5x 10^5 cells/60 mm plate 24 hours prior to transfection. The cells were washed and incubated for 30 minutes at 37°C with 2 ml of Opti-MEM media. The DNA-LipofectAMINE™-Opti-MEM mixture (8 µg of mutant DNA, 0.4µg of pJ6Ωbleo, 48 µl lipofectAMINE™, and 300 µl Opti-MEM media) was incubated for 30 minutes at 25°C. After the 30 minute incubations, 2.4 ml of Opti-MEM media was added to the DNA-LipofectAMINE™ mixture. The resulting solution was layered onto the gpGFP cells. Eight hours later the transfection mixture was removed and

the cells were incubated with DMEM FBS/PS for 40 hours before transferring the cells to 10 cm plates at two different dilutions (1/10 and 1/100). The following day, the media was changed to D-MEM FBS/PS containing 200 µg/ml of Zeocin. Colonies appeared after two weeks and were picked for screening by an infectivity assay described below. The cell lines so produced were labeled "SafeEbola-GFP".

The supernatant medium from the cells was filtered through a 0.45 µm filter and then incubated with 1 x 10^6 NIH 3T3 cells in the presence of 8 µg/ml polybrene for 4 hours. The recombinant-virus-containing medium was then replaced with D-MEM CS/PS. Forty-eight hours later the cells were removed from the plate, suspended in 1xPBS containing 1 mM EDTA, and analyzed by flow cytometry with a Coulter XL-MCL Flow Cytometer, using a 525 nm band-pass filter and a 488 nm air-cooled argon laser.

Stable cell lines that produce pseudotyped retrovirus not containing specific nucleotide sequences such as those encoding the green fluorescent protein were produced in the same manner, except the parent cell line to the gpGFP cells were used instead (i.e., ΦNX cells, human embryonic kidney cells transfected only with MMLV *gag* and *pol* nucleotide sequences). These cell lines were labeled "SafeEbola".

As seen in FIG. 4, lower panel B, cells (45.8% as determined by fluorescence activated cell sorting) transduced with pseudotyped retroviruses produced from SafeEbola-GFP cells exhibited detectable green fluorescence.

Analysis

Cell lines that stably produce MMLV virus pseudotyped with Ebola Zaire glycoprotein have been produced. The cells indefinitely produce the pseudotyped retrovirus. The glycoprotein used to form the pseudotyped retrovirus is not toxic. The cells require diligence in care (i.e., changing the media every two days) so that the pH does not drop and syncytia formation does not occur.

EXAMPLE 10

Formation of Syncytia in Stable SafeEBola-GFP Cell Lines at Acidic pH

This example shows that SafeEbola-GFP cell lines are capable of forming syncytia at acidic pH.

5 x 10^5 SafeEbola-GFP cells or ΦNX cells, obtained as described in Examples 10 and 1, respectively, were plated on 60 mm tissue-culture dishes, grown to near confluence, washed with PBS and treated with fusion buffer [PBS containing 10 mM 2-(N-morpholino)ethane sulfonic acid and 10 mM HEPES adjusted to pH 5.5] for one minute. The low pH solution was replaced with D-MEM FBS/PS, incubated in a CO_2 incubator at 37°C, and the cells were stained with Giemsa solution 5 hours after treatment and photographed. As seen in FIG. 5A, the SafeEbola-GFP cell lines form syncytia at acidic pH, whereas no such syncytia are formed in ΦNX cells as seen in FIG. 5B.

EXAMPLE 11

Generation of Cell Lines Transiently Producing Marburg Virus Glycoprotein Pseudotyped Retrovirus

Marburg Glycoprotein Expression Plasmid

Marburg plasmid pMBGP1 was constructed from a plasmid from Hans-Dieter Klenk (Marburg, Germany). To construct this plasmid, the nucleotides 5931-8033 from the Marburg virus genome [the genomic nucleotide sequence HK Klenk, as delineated in Will et al. (1993), *J. Virol.* 67:1203-1210 and as seen in Genbank Accession Number Z12132 shown in SEQ ID 3] were cloned into the pSP72 plasmid (from Promega) under the control of the T7 promoter using SalI. The XhoI and Eco RI fragment of this plasmid was cloned into the XhoI and Eco RI polylinker sites of the

mammalian expression vector pcDNA3. SEQ ID 3 also shows the amino acid sequence of the Marburg virus glycoprotein.

Transient Transfection Procedure

The transient transfection protocol was identical to that recited in Example 8 (Ebola-glycoprotein transfection protocol), with the exception that, instead of pEZGP1, 4 μg of pMBGP1 was used.

Analysis

It has been shown that cell lines may be constructed that produce MMLV that is pseudotyped with the Marburg virus glycoprotein. The cell lines were found to produce the pseudotyped retroviruses at a titer of about 1.4×10^3 TU/ml of supernatant. The cells were able to produce the virus for a period of about 24 hours. In data not shown, it was found that NIH 3T3, BHK and HeLa cells can be efficiently transduced by this inventive pseudotyped retrovirus. This demonstrates the expanded host range of the pseudotyped retroviruses, which allows these pseudotyped retroviruses to be advantageously used to introduce desired nucleotide sequences into target cells.

EXAMPLE 12

Generation of Cell Lines Stably Producing Marburg Virus Glycoprotein Pseudotyped Retrovirus

Stable Transfection Procedure

The stable transfection protocol was identical to that recited in Example 9 (Ebola-glycoprotein transfection protocol), with the exception that 4 μg of pMBGP1 (described in Example 11) was used.

Analysis

It has been shown that cell lines may be constructed that stably, and thus indefinitely, produce MMLV that is pseudotyped with the Marburg virus glycoprotein. The cell lines were found to produce the pseudotyped retroviruses at a titer of about 1.9×10^3 TU/ml of supernatant. The glycoprotein incorporated into the lipid bilayer of the pseudotyped retroviruses is not toxic. Moreover, the cells require diligence in care (i.e., changing of the media every two days) so that the pH does not drop and syncytia formation does not occur.

While the invention has been illustrated and described in detail in the drawings and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiment has been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected. In addition, all references cited herein are indicative of the level of skill in the art and are hereby incorporated by reference in their entirety.

CLAIMS

What is claimed is:

1. A eukaryotic cell, comprising:

(a) a first nucleotide sequence encoding a retroviral Gag polypeptide;

(b) a second nucleotide sequence encoding a retroviral Pro polypeptide;

(c) a third nucleotide sequence encoding a retroviral Pol polypeptide; and

(d) a fourth nucleotide sequence encoding at least two different viral glycoproteins.

2. The cell of claim 1, wherein said cell further comprises a fifth nucleotide sequence having a 5' and a 3' end, said fifth nucleotide sequence encoding a desired protein, said fifth nucleotide sequence operably linked at said 5' end to a first retroviral long terminal repeat sequence and operably linked at said 3' end to a second retroviral long terminal repeat sequence.

3. The cell of claim 2, wherein said desired protein is a marker.

4. The cell of claim 3, wherein said marker is a fluorescent protein.

5. The cell of claim 1, wherein said two different viral glycoproteins are togaviral glycoproteins.

6. The cell of claim 5, wherein said togaviral glycoproteins are alphaviral glycoproteins.

7. The cell of claim 6, wherein said alphaviral glycoprotein is a Ross River alphaviral glycoprotein.

8. The cell of claim 1, wherein said eukaryotic cell is a mammalian cell.

9. The cell of claim 8, whererin said mammalian cell is a human cell.

10. The cell of claim 1, wherein said retroviral Gag, Pol and Pro polypeptides are comprised of Moloney murine leukemia Gag, Pro and Pol polypeptides.

11. The cell of claim 1, wherein said cell produces a pseudotyped retrovirus having a lipid bilayer, said viral glycoproteins disposed in said lipid bilayer.

12. The cell of claim 1, wherein said first, second, third and fourth nucleotide sequences are chromosomally-integrated.

13. A eukaryotic cell, comprising:

(a) a first nucleotide sequence encoding a retroviral Gag polypeptide;

(b) a second nucleotide sequence encoding a retroviral Pro polypeptide;

(c) a third nucleotide sequence encoding a retroviral Pol polypeptide; and

(d) a fourth nucleotide sequence encoding a filoviral glycoprotein, said first, second, third and fourth nucleotide sequences being chromosomally-integrated, said cell stably producing pseudotyped retroviruses.

14. The cell of claim 13, wherein said cell further comprises a fifth nucleotide sequence having a 5' end and a 3' end, said fifth nucleotide sequence encoding a desired protein, said fifth nucleotide sequence operably linked at said 5' end to a first retroviral long terminal repeat sequence and operably linked at said 3' end to a second retroviral long terminal repeat sequence.

15. The cell of claim 13, wherein said filoviral glycoprotein is selected from the group consisting of Marburg virus glycoprotein and Ebola virus glycoprotein.

16. The cell of claim 13, wherein said retroviral Gag, Pro and Pol polypeptides are comprised of Moloney murine leukemia virus Gag, Pro and Pol polypeptides.

17. The cell of claim 13, wherein said cell produces pseudotyped retrovirus at a titer of at least about 4.5×10^4 transforming units/ml of supernatant.

18. A eukaryotic cell, comprising:

(a) a first nucleotide sequence encoding a retroviral Gag polypeptide;

(b) a second nucleotide sequence encoding a retroviral Pro polypeptide;

(c) a third nucleotide sequence encoding a retroviral Pol polypeptide; and

(d) a fourth nucleotide sequence encoding a Marburg virus glycoprotein.

19. A method of forming a eukaryotic cell for producing pseudotyped retroviruses, said method comprising:

transfecting a eukaryotic cell with a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least two different viral glycoproteins.

20. The method of claim 19, wherein said first, second and third nucleotide sequences are operably linked to a promoter sequence.

21. The method of claim 19, wherein said viral glycoproteins are togaviral glycoproteins.

22. The method of claim 21, wherein said togaviral glycoproteins are alphaviral glycoproteins.

23. The method of claim 22, wherein said alphaviral glycoproteins are Ross River alphaviral glycoproteins.

24. The method of claim 19, wherein said first, second, third and fourth nucleotide sequences are chromosomally-integrated.

25. The method of claim 19, wherein said cell further comprises a fifth nucleotide sequence having a 5' end and a 3' end, said fifth nucleotide sequence encoding a desired protein, said fifth nucleotide sequence operably linked at said 5' end to a first retroviral long terminal repeat sequence and operably linked at said 3' end to a second retroviral long terminal repeat sequence.

26. A method of forming a eukaryotic cell for producing pseudotyped retroviruses, said method comprising:

(a) transfecting a eukaryotic cell with a vector including a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide and a third nucleotide sequence encoding a retroviral Pol polypeptide, said first, second and third nucleotide sequences operably linked to a first promoter sequence; and

(b) transfecting said cell with a fourth nucleotide sequence encoding at least two viral glycoproteins, said fourth nucleotide sequence operably linked to a second promoter sequence.

27. The method of claim 26, said method further comprising transfecting said cell with a vector including a fifth nucleotide sequence having a 5' and a 3' end, said fifth nucleotide sequence encoding a desired protein, said fifth nucleotide sequence operably linked at said 5' end to a first retroviral long terminal repeat sequence and operably linked at said 3' end to a second retroviral long terminal repeat sequence.

28. The method of claim 26, wherein said desired protein is a marker.

29. The method of claim 26, wherein said first, second, third and fourth nucleotide sequences are chromosomally-integrated.

30. A method of forming a eukaryotic cell for producing pseudotyped retroviruses, said method comprising:

(a) transfecting a eukaryotic cell with a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding a

filoviral glycoprotein, said first, second, third and fourth nucleotide sequences being chromosomally-integrated, said cell stably producing pseudotyped retroviruses.

31. The method of claim 30, wherein said filoviral glycoprotein is selected from the group consisting of Ebola virus glycoprotein and Marburg virus glycoprotein.

32. A method of forming a eukaryotic cell for producing pseudotyped retroviruses, said method comprising:

transfecting a eukaryotic cell with a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding a Marburg virus glycoprotein.

33. A pseudotyped retrovirus, comprising:

(a) a retroviral capsid;

(b) a lipid bilayer; said lipid bilayer surrounding said retroviral capsid; and

(c) at least two different viral glycoproteins disposed in said lipid bilayer.

34. The retrovirus of claim 33, said retrovirus further comprising a nucleotide sequence encoding a desired protein, said nucleotide sequence enclosed within said retroviral capsid.

35. The retrovirus of claim 33, wherein said viral glycoproteins are togaviral glycoproteins.

36. The retrovirus of claim 35, wherein said togaviral glycoproteins are alphaviral glycoproteins.

37. The retrovirus of claim 36, wherein said alphaviral glycoproteins are Ross River alphaviral glycoproteins.

38. The retrovirus of claim 33, wherein said retroviral capsid is comprised of a Moloney murine leukemia virus capsid.

39. A pseudotyped retrovirus, comprising:

(a) a retroviral capsid;

(b) a lipid bilayer; said lipid bilayer surrounding said retroviral capsid; and

(c) a Marburg virus glycoprotein disposed in said lipid bilayer.

40. A method of introducing a nucleotide sequence into a cell, said method comprising:

transducing a cell permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer with a pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer; said lipid bilayer surrounding said retroviral capsid;

at least two different viral glycoproteins disposed in said lipid bilayer; and

a desired ribonucleotide sequence.

41. The method of claim 40, wherein said retroviral capsid is a Moloney murine leukemia virus capsid.

42. The method of claim 40, wherein said virus having at least two different glycoproteins in its lipid bilayer is a togavirus, and said at least two different viral glycoproteins are togaviral glycoproteins.

43. The method of claim 42, wherein said togavirus is an alphavirus and said togaviral glycoproteins are alphaviral glycoproteins.

44. A method of introducing a nucleotide sequence into a cell, said method comprising:

transducing a cell permissive for Marburg virus entry with a pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer; said lipid bilayer surrounding said retroviral capsid;

a Marburg virus glycoprotein disposed in said lipid bilayer; and

a desired ribonucleotide sequence.

45. A method of screening agents effective in blocking viral entry into a cell, said method comprising:

(a) treating a pseudotyped retrovirus with said agent, said pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer, said lipid bilayer surrounding said retroviral capsid;

at least two different viral glycoproteins disposed in said lipid bilayer; and

a nucleotide sequence encoding a desired marker, said nucleotide sequence enclosed within said retroviral capsid;

(b) treating a cell permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer with said treated pseudotyped retrovirus; and

(c) identifying eukaryotic cells having the desired marker.

46. The method of claim 45, wherein said virus having at least two different viral glycoproteins disposed in its lipid bilayer is a togavirus and said two different viral glycoproteins are togaviral glycoproteins.

47. The method of claim 46, wherein said togavirus is an alphavirus and said togaviral glycoproteins are alphaviral glycoproteins.

48. The method of claim 45, wherein said agent is an immunological agent.

49. The method of claim 45, wherein said agent is a pharmacological agent.

50. A method of screening agents effective in blocking Marburg virus entry into a cell, said method comprising:

(a) treating a pseudotyped retrovirus with said agent, said pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer, said lipid bilayer surrounding said retroviral capsid;

a Marburg virus glycoprotein disposed in said lipid bilayer; and

a nucleotide sequence encoding a desired marker, said nucleotide sequence enclosed within said retroviral capsid;

(b) treating a cell permissive for Marburg virus entry with said treated pseudotyped retrovirus; and

(c) identifying eukaryotic cells having the desired marker.

51. A method of screening agents effective in blocking viral entry into a cell, said method comprising:

(a) treating a cell permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer with said agent;

(b) contacting said treated cell with a pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer, said lipid bilayer surrounding said retroviral capsid;

at least two different viral glycoproteins disposed in said lipid bilayer;

a nucleotide sequence encoding a desired marker, said nucleotide sequence enclosed within said retroviral capsid; and

(c) identifying eukaryotic cells having the desired marker.

52. A method of screening agents effective in blocking viral entry into a cell, said method comprising:

(a) treating a cell permissive for entry of a Marburg virus with said agent;

(b) contacting said treated cell with a pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer, said lipid bilayer surrounding said retroviral capsid;

a Marburg virus glycoprotein disposed in said lipid bilayer;

a nucleotide sequence encoding a desired marker, said nucleotide sequence enclosed within said retroviral capsid; and

(c) identifying eukaryotic cells having the desired marker.

53. A kit for forming a pseudotyped retrovirus, said kit comprising:

(a) a first nucleotide sequence encoding a retroviral Gag polypeptide;

(b) a second nucleotide sequence encoding a retroviral Pro polypeptide;

(c) a third nucleotide sequence encoding a retroviral Pol polypeptide; and

(d) a fourth nucleotide sequence encoding at least two different viral glycoproteins.

54. The method of claim 52, wherein said viral glycoproteins are togaviral glycoproteins.

55. A kit for forming a pseudotyped retrovirus, said kit comprising:

(a) a first nucleotide sequence encoding a retroviral Gag polypeptide;

(b) a second nucleotide sequence encoding a retroviral Pro polypeptide;

(c) a third nucleotide sequence encoding a retroviral Pol polypeptide; and

(d) a fourth nucleotide sequence encoding a Marburg virus glycoprotein.

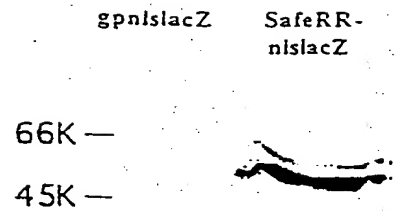


FIG. 1

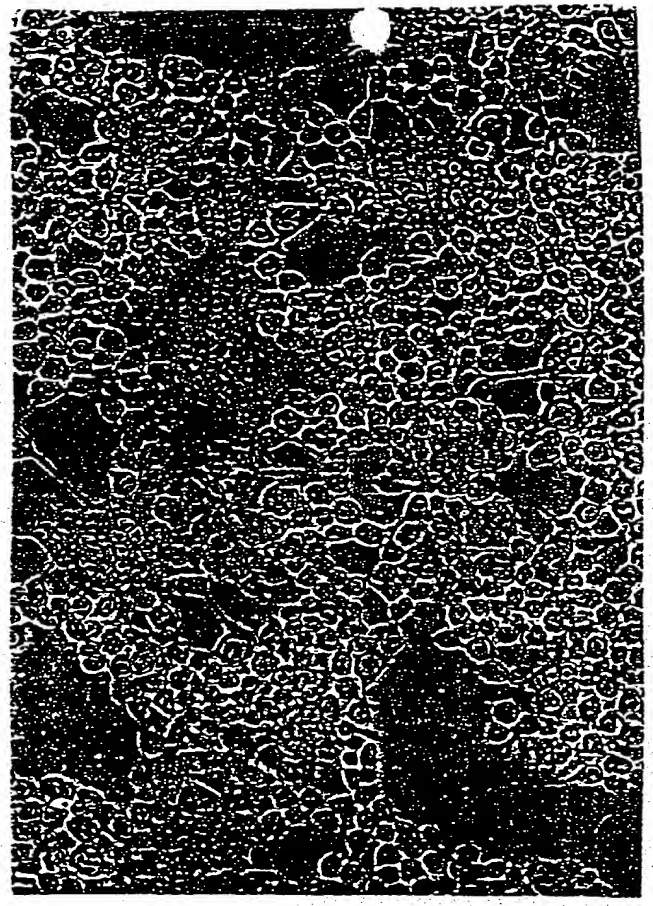


Fig 2 A

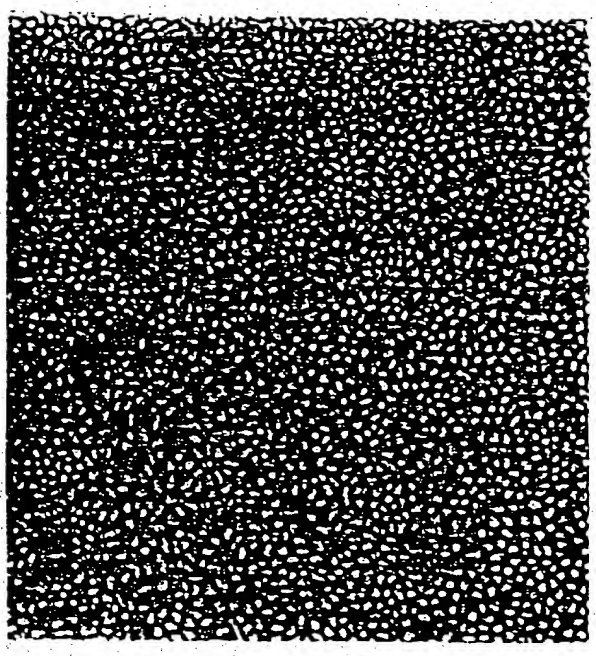


Fig. 2 B

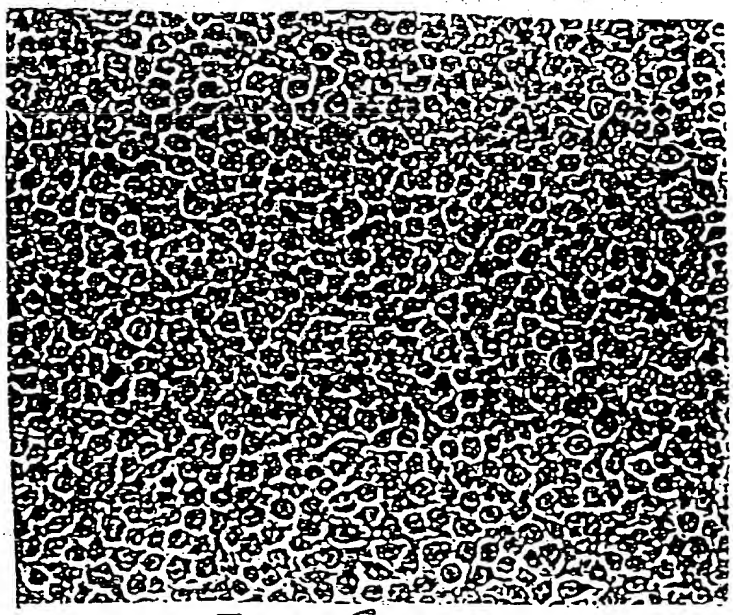


Fig. 2 C

Fig. 3 A

Fig. 3 B

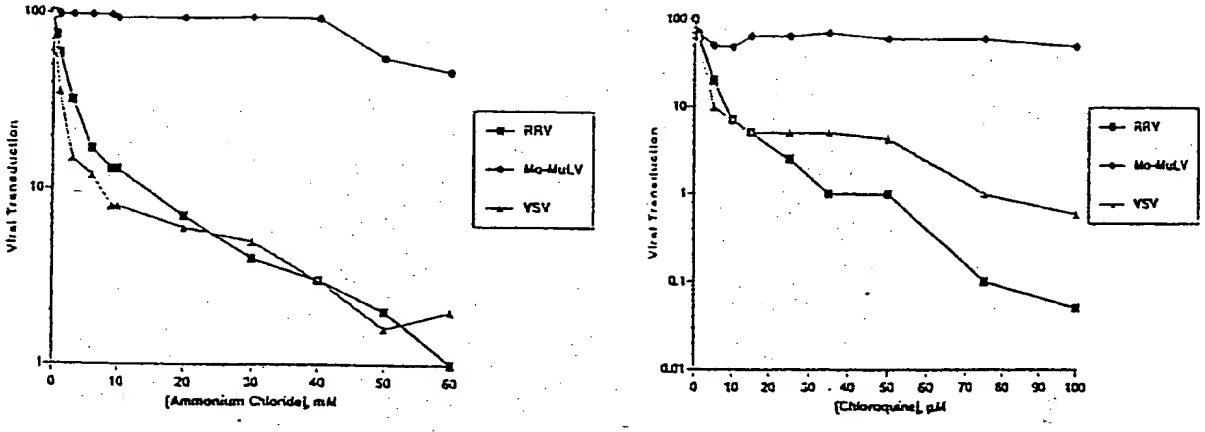


Fig 4 A

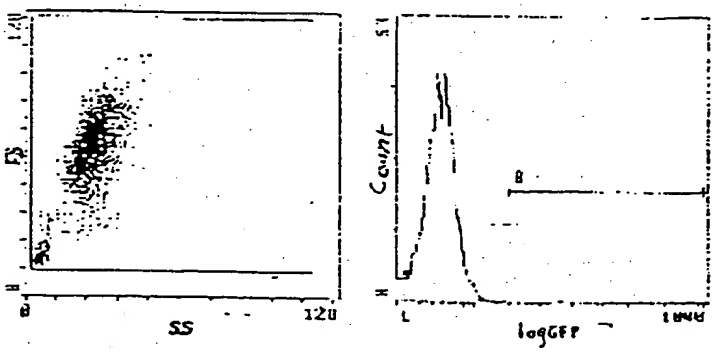


Fig 4 B

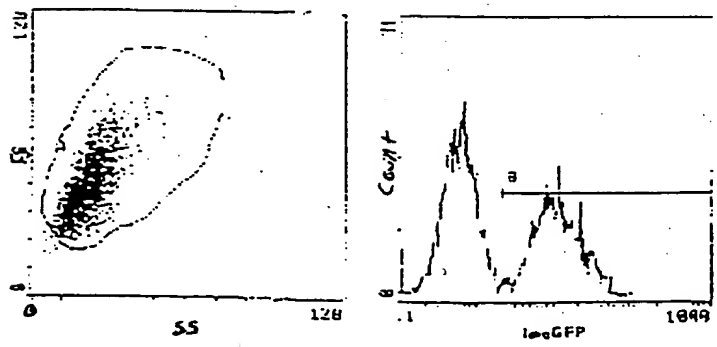


Fig5 A

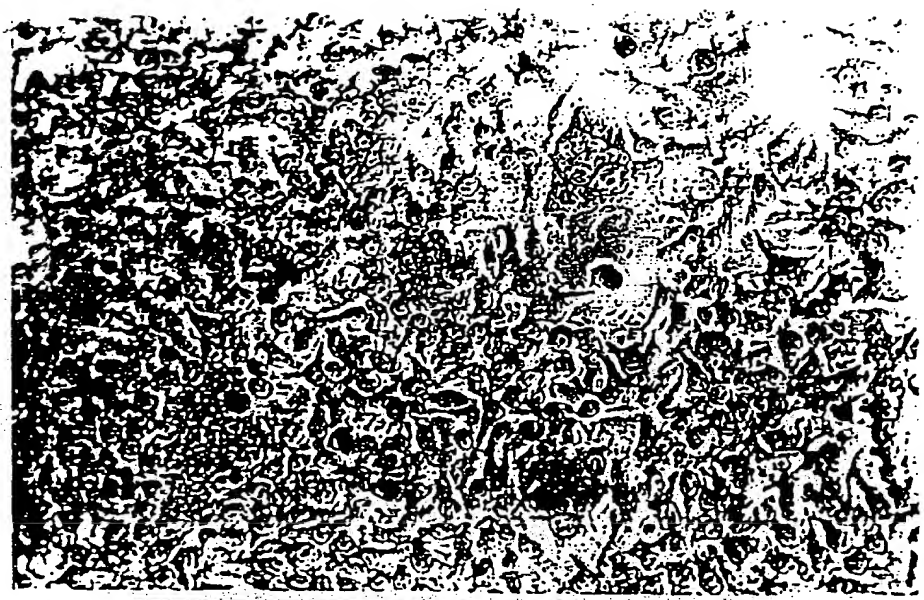
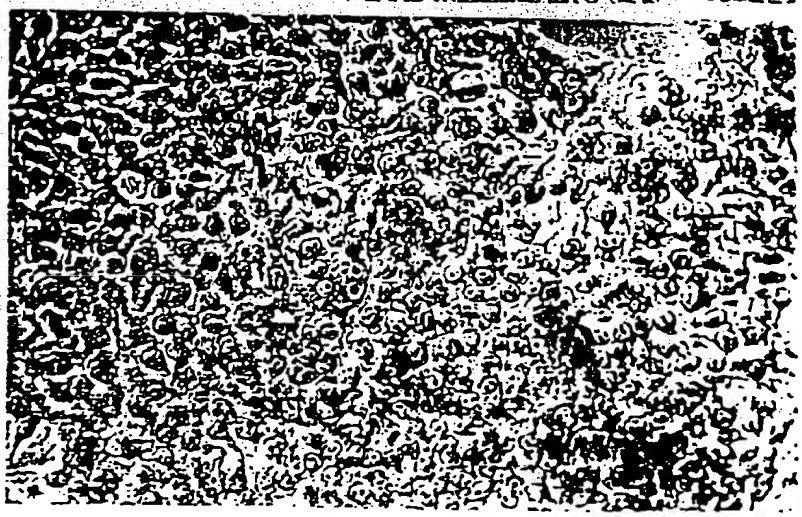


Fig. 5B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17702

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C12P 21/06; C12N 7/04, 5/00; A61K 39/12
US CL :435/69.1, 236, 325; 424/199.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 236, 325; 424/199.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPATFUL, MEDLINE, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,491,084 A (CHALFIE et al.) 13 February 1996, see entire document.	3-4
Y	US 5,512,421 A (BURNS et al.) 30 April 1996, see entire document.	1-12
Y	US 5,591,624 A (BARBER et al.) 07 January 1997, see entire document.	1-12
Y	US 5,503,974 A (GRUBER et al.) 02 April 1996, see entire document.	1-12
Y	US 5,723,287 A (RUSSELL et al.) 03 March 1998, see entire document.	1-12
Y	US 5,278,056 A (BANK et al.) 11 January 1994, see entire document.	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search	Date of mailing of the international search report
25 JANUARY 2000	10 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Jeffrey S. Parkin, Ph.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17702

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,185,440 A (DAVIS et al.) 09 February 1993, see entire document.	5-7
Y	LOPEZ, S. et al. Nucleocapsid-Glycoprotein Interactions Required for Assembly of Alphaviruses. J. Virol. March 1994, Vol. 68, No. 3, pages 1316-1323, see entire document.	5-7
Y	KUHN, R. J. et al. Chimeric Sindbis-Ross River Viruses to Study Interactions between Alphavirus Nonstructural and Structural Regions. J. Virol. November 1996, Vol. 70, No. 11, pages 7900-7909, see entire document.	5-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17702

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-12

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17702

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-12, drawn to a eukaryotic cell comprising nucleotide sequences encoding, inter alia, at least two different viral glycoproteins.
Group II, claim(s) 13-18, drawn to a eukaryotic cell comprising nucleotide sequences encoding, inter alia, a filoviral glycoprotein.
Group III, claim(s) 19-29, drawn to a method of producing a eukaryotic cell capable of producing pseudotyped retroviruses with two different viral glycoproteins.
Group IV, claim(s) 30-32, drawn to a method of producing a eukaryotic cell capable of producing pseudotyped retroviruses with a filoviral glycoprotein.
Group V, claim(s) 33-38, drawn to a pseudotyped retrovirus containing at least two different viral glycoproteins.
Group VI, claim(s) 39, drawn to a pseudotyped retrovirus containing a Marburg virus glycoprotein.
Group VII, claim(s) 40-43, drawn to a method of introducing a nucleotide sequence into a cell by transducing a cell with a pseudotyped retrovirus expressing at least two different viral glycoproteins.
Group VIII, claim(s) 44, drawn to a method of introducing a nucleotide sequence into a cell by transducing a cell with a pseudotyped retrovirus expressing a Marburg virus glycoprotein.
Group IX, claim(s) 45-49 and 51, drawn to a method of screening for agents effective in blocking viral entry employing a pseudotyped retrovirus expressing at least two different viral glycoproteins.
Group X, claim(s) 50, 52, and 54, drawn to a method of screening agents effective in blocking Marburg virus entry into a cell employing a pseudotyped retrovirus expressing a Marburg virus glycoprotein.
Group XI, claim(s) 53, drawn to a kit for forming pseudotyped retroviruses containing at least two different viral glycoproteins.
Group XII, claim(s) 55, drawn to a kit for forming psuedotyped retroviruses containing a Marburg virus glycoprotein.

The inventions listed as Groups I-XII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The claims are directed toward multiple products (e.g., eukaryotic cells, pseudotyped retroviral particles, kits) with different chemical structures/compositions and attendant features (e.g., expressing two different viral glycoproteins, expressing a single virus glycoprotein). The claims are also directed toward multiple methods (e.g., method of making a eukaryotic cell capable of producing retroviral pseudotypes, method of gene transduction employing pseudotyped retroviral particles, method of screening for putative antiviral agents) that employ different reagents, methodology steps, and accomplish different scientific objectives. Accordingly, the claims all lack a special technical feature and are directed toward different inventive concepts.

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

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(54) Title: PSEUDOTYPED RETROVIRUSES AND STABLE CELL LINES FOR THEIR PRODUCTION

(57) Abstract

Cells that produce inventive pseudotyped retroviruses having a broad host range have been produced. In one aspect of the invention, the cells produce retroviruses pseudotyped with at least two different viral glycoproteins, such as togaviral glycoproteins. In alternative embodiments, the cells produce retroviruses pseudotyped with filoviral glycoproteins. Methods of producing the above-described cells, as well as the pseudotyped retroviruses thus produced, are also provided. In other embodiments, methods of screening agents effective in blocking viral entry into a cell, including filoviral entry or entry of viruses having at least two different viral glycoproteins disposed in their lipid bilayer, such as togaviruses, are provided. Moreover, methods of using the inventive pseudotyped retroviruses for introducing nucleotide sequences into target cells, and kits for forming the inventive pseudotyped retroviruses, are also provided.

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PSEUDOTYPED RETROVIRUSES AND STABLE CELL LINES FOR THEIR PRODUCTION

REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Patent Application Serial Number 60/095,242, filed on August 4, 1998, and U.S. Patent Application Serial Number 60/112,405, filed on December 15, 1998, which are both hereby incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

The present invention relates generally to cells that produce pseudotyped retroviruses having broad host range. Specifically, the invention relates to cells that produce retroviruses pseudotyped with glycoproteins derived from either filoviruses or viruses having at least two different viral glycoproteins disposed in their lipid bilayer. The invention further relates to methods of producing such cells, the pseudotyped retroviruses produced, methods of making and using the pseudotyped retroviruses and kits for producing the pseudotyped retroviruses.

Retroviruses are ribonucleic acid (RNA) viruses that include an RNA genome enclosed within a viral capsid wherein the capsid is surrounded by an envelope, or lipid bilayer. Glycoproteins present in the lipid bilayer (envelope glycoproteins) interact with receptors on the surface of various host cells and allow the retroviruses to enter the host cell. Once in the cell, the retroviruses reverse transcribe the RNA of the viral genome into a double-stranded DNA (a proviral intermediate), and incorporate the deoxyribonucleic acid (DNA) into the cellular genome as a provirus. Gene products from the integrated foreign DNA may then be produced so that progeny viral particles may be assembled. As retroviruses can be modified to carry exogenous nucleotide sequences of interest, such recombinant retroviruses have a variety of uses. For example, such recombinant retroviruses are important in introducing desired exogenous

sequences into a cell, so that relatively high levels of the protein encoded by the sequences may be produced. However, use of such recombinant retroviruses has several drawbacks.

For example, retroviruses do not have a broad host range. Efforts at increasing the host range of retroviruses have included substituting the envelope glycoproteins of the virus with that of a different virus, thus forming a pseudotyped retrovirus. The pseudotyped retrovirus advantageously has the host range of the different virus. However, some retroviruses have been pseudotyped with viral glycoproteins that are toxic to cells, so the cells can only produce the virus for a limited time. Furthermore, in many cases, the pseudotyped retroviruses can not be stably produced and may not be produced at a high titer.

There is therefore a need for pseudotyped retroviruses of broad host range, and cell lines capable of producing such pseudotyped retroviruses. The present invention addresses this need.

SUMMARY OF THE INVENTION

It has been discovered that cells may be constructed to produce inventive retroviruses pseudotyped with viral glycoproteins, wherein the retroviruses have a broad host range. Accordingly, one aspect of the invention provides eukaryotic cells that include a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one preferred embodiment, the fourth nucleotide sequence encodes at least two different viral glycoproteins, preferably togaviral glycoproteins, such as, for example, alphaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a filoviral glycoprotein, such as, for example, a Marburg virus or Ebola virus glycoprotein. In a preferred form of the invention, the cells stably produce inventive pseudotyped retroviruses.

A second aspect of the invention provides methods of forming the above-described eukaryotic cells. The method includes transfecting a eukaryotic cell with a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one preferred embodiment, the fourth nucleotide sequence encodes at least two different viral glycoproteins, preferably togaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a filoviral glycoprotein, such as a Marburg virus glycoprotein. In preferred forms of the invention, the first, second, third and fourth nucleotide sequences are chromosomally-integrated, wherein the cell stably produces inventive pseudotyped retroviruses.

A third aspect of the invention provides inventive pseudotyped retroviruses, including a retroviral capsid, a lipid bilayer surrounding the retroviral capsid and at least one viral glycoprotein disposed in the lipid

bilayer. In inventive pseudotyped retroviruses, at least two different viral glycoproteins are disposed in the lipid bilayer, and in preferred embodiments, the viral glycoproteins are togaviral glycoproteins. In an alternative embodiment, the viral glycoprotein is a filoviral glycoprotein, preferably a Marburg virus glycoprotein.

In yet a fourth aspect of the present invention, methods of introducing nucleotide sequences into a cell are provided, and include transducing a cell permissive for viral entry with a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer surrounding the retroviral capsid, at least one viral glycoprotein disposed in the lipid bilayer and a desired ribonucleotide sequence. In one preferred form of the invention, the cells are permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer, such as a togavirus wherein the viral glycoproteins are togaviral glycoproteins. In alternative embodiments, the viral glycoprotein is a filoviral glycoprotein, preferably a Marburg virus glycoprotein.

A fifth aspect of the invention provides methods of screening agents effective in blocking viral entry into a cell. In one mode of practicing the invention, the method includes treating a pseudotyped retrovirus with the agent, treating a cell permissive for viral entry with the treated pseudotyped retrovirus and identifying eukaryotic cells having the desired marker. In one embodiment, the pseudotyped retrovirus has a retroviral capsid, a lipid bilayer surrounding the capsid, at least two different viral glycoproteins disposed in its lipid bilayer, such as togaviral glycoproteins wherein the cell is permissive for togaviral entry, and a nucleotide sequence encoding a desired marker. In alternative embodiments, a method is provided for screening agents effective in blocking filoviral entry, preferably Marburg virus entry, into a cell. Pseudotyped retroviruses having Marburg virus glycoprotein disposed in their lipid bilayer are preferred as are cells permissive for Marburg virus entry.

In yet another embodiment of a method of screening agents effective in blocking viral entry into a cell, the method includes treating a cell permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer with said agent, contacting the treated cell with a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer that surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer, such as togaviral glycoproteins wherein the cell is permissive for togaviral entry, and a nucleotide sequence encoding a desired marker, and identifying cells having the marker. In alternative embodiments, a method is provided for screening agents effective in blocking filoviral entry, preferably Marburg virus entry, into a cell. Pseudotyped retroviruses having Marburg virus glycoprotein disposed in their lipid bilayer are preferred as are cells permissive for Marburg virus entry.

In a sixth aspect of the present invention, kits for forming inventive pseudotyped retroviruses are provided. The kits include a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one embodiment, the fourth nucleotide sequence encodes at least two viral glycoproteins, such as togaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a Marburg virus glycoprotein.

One object of the invention is to provide a eukaryotic cell including a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein, such as a Marburg virus glycoprotein, preferably at least two viral glycoproteins, such as togaviral glycoproteins and especially alphaviral glycoproteins.

Another object is to provide a eukaryotic cell that includes a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein, such as a Marburg virus glycoprotein, preferably at least two viral glycoproteins, such as togaviral glycoproteins and especially alphaviral glycoproteins, wherein the cell stably produces the inventive pseudotyped retroviruses.

Another object is to provide a method of making the inventive cells described above, as well as the pseudotyped retroviruses so produced.

Other objects are to provide a method of screening agents effective in blocking either filoviral entry into a cell or entry of viruses having more than one viral glycoprotein in their lipid bilayer, such as togaviruses, and methods of introducing desired nucleotide sequences into a cell.

Yet other objects of the invention are to provide kits for forming inventive pseudotyped retroviruses.

These and other objects and advantages of the present invention will be apparent from the descriptions herein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows a Western blot of proteins derived from lysates of stable cell line SafeRRnlslacZ, or precursor gpnlslacZ cells, as further described in Example 4.

FIG. 2 depicts Giemsa solution-stained SafeRR-nlslacZ cells (Panel A, FIG. 2A) and ΦNX cells (Panel B, FIG. 2B) after being incubated at room temperature for one hour with pH 5.5 fusion buffer and grown in D-MEM FBS/PS culture medium for four hours as described in Example 5. Panel C (FIG. 2C) depicts Giemsa solution-stained SafeRR-nlslacZ cells treated in a similar manner with the exception that they were exposed to pH 7 fusion buffer instead of pH 5.5 fusion buffer.

FIG. 3 depicts graphs showing the effects of lysosomotropic agents on transduction of the indicated retroviruses. Left panel, A, FIG. 3A, shows the effect of ammonium chloride and right panel, B, FIG. 3B, shows the effect of chloroquine. RRV, pseudotyped virus obtained from supernatants of SafeRR-nlslacZ cells; Mo-MuLV, wild type Moloney murine leukemia virus expressing the env glycoprotein; VSV;Moloney murine leukemia virus pseudotyped with vesicular stomatitis viral glycoprotein G.

FIG. 4 shows fluorescence profiles of NIH 3T3 cells transduced with supernatant medium from ΦNX cells (top panel, A, FIG. 4A) or Safe-Ebola-GFP cells (bottom panel, B, FIG. 4B) according to the procedure outlined in Example 9.

FIG. 5 depicts syncytia formation by packaging cells expressing Ebola glycoprotein. The cells were treated according to the protocol in Example 10. Top panel, A, (FIG. 5A) SafeEbola-GFP cells; Bottom panel, B, FIG. 5B, ΦNX cells.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to preferred embodiments and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations and further modifications of the invention, and such further applications of the principles of the invention as illustrated herein, being contemplated as would normally occur to one skilled in the art to which the invention relates.

The present invention relates to eukaryotic cells that stably produce pseudotyped retroviruses and methods for their production, pseudotyped retroviruses, methods of introducing nucleotide sequences into a target cell, methods of screening agents effective in blocking viral entry into cells and kits for forming inventive pseudotyped retroviruses.

It has been discovered that eukaryotic cells may be constructed that either transiently or stably produce pseudotyped retroviruses having at least two different viral glycoproteins disposed in their lipid bilayer, such as togaviral glycoproteins. It has further been discovered that eukaryotic cells may be constructed that stably produce pseudotyped retroviruses having filoviral glycoproteins disposed in their lipid bilayer. The pseudotyped retroviruses of the present invention are advantageous in transducing cells of interest, are not toxic to the cells, have a broad host range and do not allow for pseudotransduction (i.e., introduction of proteins and/or genetic material without stable transmission of genetic material). Moreover, the present disclosure is the first report of a pseudotyped retrovirus having two different viral glycoproteins, with different membrane spanning domains, disposed in its lipid bilayer.

Accordingly, one aspect of the invention provides inventive eukaryotic cells having nucleotide sequences encoding retroviral Gag polypeptide, retroviral Pro polypeptide, retroviral Pol polypeptide and at

least one viral glycoprotein, such as a filoviral glycoprotein, or at least two viral glycoproteins, such as togaviral glycoproteins. In a preferred embodiment, nucleotide sequences encoding the polypeptides described are chromosomally-integrated and thus stably produce inventive pseudotyped retroviruses. A second aspect of the invention provides methods of forming cells that produce inventive pseudotyped retroviruses. A third aspect of the invention provides the inventive pseudotyped retroviruses, preferably those that include at least two different viral glycoproteins disposed in their lipid bilayer, including togaviral glycoproteins, and further preferably those that include a desired nucleotide sequence in their genome. Other aspects of the invention provide inventive methods of introducing a nucleotide sequence into a desired cell and methods of screening agents effective in blocking viral entry into a target cell, preferably blocking entry of a Marburg virus, or a virus having more than one viral glycoprotein in its lipid bilayer such as a togavirus, wherein all of the methods utilize the inventive pseudotyped retroviruses and cells described above, and kits for producing inventive pseudotyped retroviruses.

As discussed above, one aspect of the invention provides eukaryotic cells, forming inventive eukaryotic cell lines, having nucleotide sequences encoding retroviral Gag polypeptide, retroviral Pro polypeptide, retroviral Pol polypeptide and at least one viral glycoprotein, such as a filoviral glycoprotein, or at least two different viral glycoproteins, typically from the same virus, such as togaviral glycoproteins. The term "eukaryotic cell line" as used herein is intended to refer to eukaryotic cells that are grown *in vitro*. The term "nucleotide sequence", as used herein, is intended to refer to a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, and derivatives thereof. The terms "encoding" and "coding" refer to the process by which a nucleotide sequence, through the mechanisms of transcription and translation, provides the information to a

cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce a polypeptide.

In forming a cell that produces an inventive pseudotyped retrovirus, a wide variety of cells may be selected. Eukaryotic cells are preferred, whereas mammalian cells are more preferred, and include human, simian canine, feline, equine and rodent cells. Human cells are most preferred. It is further preferred that the cell be able to reproduce indefinitely, and is therefore immortal. Examples of cells that may be advantageously used in the present invention include NIH 3T3 cells, COS cells, Madin-Darby canine kidney cells and human embryonic 293T cells. However, highly transfectable cells, such as human embryonic kidney 293T cells, are preferred. By "highly transfectable" it is meant that at least about 50%, more preferably at least about 70% and most preferably at least about 80% of the cells can express the genes of the introduced DNA.

The retroviral *gag*, *pro* and *pol* nucleotide sequences, and other retroviral nucleotide sequences for forming the specified pseudotyped retroviruses, may be obtained from a wide variety of genera in the family Retroviridae, including, for example, Oncoviruses, including Oncovirus A, B, C and D, lentiviruses and spumavirus F. Such sequences are preferably obtained from the Moloney murine leukemia virus (MMLV; in the genus Oncovirus C). Such sequences are well known in the art. For example, nucleotide sequences encoding MMLV gag, pro and pol may be found in Bereven et al., *Cell* (1981) 27:97-108. Most preferably, such sequences are obtained from lentiviruses. Unlike most retroviruses, lentiviruses have the capacity to integrate the genetic material they carry into the chromosomes of non-dividing cells as well as dividing cells. Therefore, lentiviral nucleotide sequences encoding proteins that allow for chromosomal integration of virally transported nucleic acid in non-dividing cells are advantageously employed, as the host range of the pseudotyped retroviruses will be broadened.

The above-described retroviruses are readily publicly available from the American Type Culture Collection (ATCC) and the desired nucleotide sequences may be obtained from these retroviruses by methods known to the skilled artisan. For example, the nucleotide sequences may be obtained by recombinant DNA technology. Briefly, viral DNA libraries may be constructed and the nucleotide sequences may be obtained by standard nucleic acid hybridization or polymerase chain reaction (PCR) procedures, using appropriate probes or primers. Alternatively, supernatant medium from cells infected with the respective virus can be isolated and the desired retroviral nucleotide sequences may be amplified by PCR. Such vectors may also be constructed by other methods known to the art.

It is preferred that the *gag*, *pro* and *pol* nucleotide sequences are contiguous to each other as found in native retroviral genomes, such as in the order 5'-gag-pro-pol-3'. It is further preferred that these retroviral nucleotide sequences are chromosomally-integrated into the cellular genome. Furthermore, the gag-pro-pol nucleotide sequences are operably linked at the 5' end of the *gag* nucleotide sequence to a promoter sequence, so that transcription of the sequences may be achieved.

A nucleic acid sequence is "operably linked" to another nucleic acid sequence, such as a promoter sequence, when it is placed in a specific functional relationship with the other nucleic acid sequence. The functional relationship between a promoter and a desired nucleic acid typically involves the nucleic acid and the promoter sequences being contiguous such that transcription of the nucleic acid sequence will be facilitated. Two nucleic acid sequences are further said to be operably linked if the nature of the linkage between the two sequences does not (1) result in the introduction of a frame-shift-mutation; (2) interfere with the ability of the promoter region sequence to direct the transcription of the desired nucleotide sequence, or (3) interfere with the ability of the desired nucleotide sequence to be transcribed by the promoter sequence region.

Typically, the promoter element is generally upstream (i.e., at the 5' end) of the nucleic acid coding sequence.

A wide variety of promoters are known in the art, including cell-specific promoters, inducible promoters, and constitutive promoters. The promoters may further be selected such that they require activation by activating elements known in the art, so that production of the protein encoded by the specified nucleic acid sequence may be regulated as desired. It is well within the purview of a person skilled in the art to select and use an appropriate promoter in accordance with the present invention. For example, the promoters that may be advantageously present in the cell, 5' to the gag-pro-pol sequences, include rat actin promoter and the MMLV promoter. Furthermore, the cytomegalovirus promoter has been found to be an excellent promoter in the inventive system.

Other regulatory elements, such as enhancer sequences, which cooperate with the promoter and transcriptional start site to achieve transcription of the nucleic acid insert coding sequence, may also be present in the cell 5' to the nucleotide sequences that encode retroviral proteins. By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity in a cell, such as a bacterial or eukaryotic host cell.

A wide variety of viral glycoproteins may be advantageously present in the inventive cells of the present invention, especially viral glycoproteins necessary for attachment of the virus to a target cell and penetration of the virus into the cytoplasm of the cell, as well as viral glycoproteins necessary for maturation of the glycoproteins necessary for attachment and penetration of the virus. For example, the cells described above may include nucleotide sequences encoding at least two different viral glycoproteins. Examples of such viruses include viruses in the families Togaviridae (e.g., in the genus *Alphavirus* or *Rubivirus*), Flaviviridae (e.g., *Flavivirus*, *Pestivirus* and *Hepatitic C*), Paramyxoviridae (e.g., Morbillivirus), and Bunyaviridae (e.g., Hantavirus). Such nucleotide sequences are well

known to the art. In one embodiment, the cells may include, instead of the viral nucleotide sequences encoding at least two different viral glycoproteins, nucleotide sequences encoding filoviral glycoproteins. Examples of such viruses include Ebola virus (including Ebola Zaire, Ebola Reston and Ebola Sudan sequences which are chromosomally-integrated), and Marburg virus. These nucleotide sequences may be obtained by methods known in the art as recited in example 2. For example, nucleotide sequences encoding particular glycoproteins may be isolated and cloned into plasmids by standard techniques, and the nucleotide sequence may then be amplified by PCR using the appropriate primers.

In one form of the present invention, the cells include nucleotide sequences encoding glycoproteins from an alphavirus. In a most preferred embodiment, the cells include nucleotide sequences encoding glycoproteins from the viral species Ross River (depicted in SEQ ID 1). The viral transmembrane glycoprotein complex that is responsible for the binding of the alphavirus to the surface of a susceptible cell and for the fusion of the viral and cellular membranes that occurs during the process of viral entry includes a trimer of a heterodimer of two transmembrane proteins, which are denoted E_1 and E_2 and which are encoded by an E_3-E_2-6K-E_1 glycoprotein coding region (E_3 and 6K refer to viral proteins involved in maturation of E_1 and E_2 as known in the art) on the alphaviral genome. The E_2-E_1 coding region includes an E_3 glycoprotein coding region as well as the 6K protein coding region. Such nucleotide sequences may be obtained by methods known to the skilled artisan as discussed for the *gag*, *pro* and *pol* nucleotide sequences above. For example, the E_2-E_1 coding region may be obtained as discussed in Kuhn et al. (1991) *Virology* 182:430-441. The E_2-E_1 glycoprotein coding region is also operably linked to a promoter sequence, such as described above, at its 5' end.

The eukaryotic cells described above, that include nucleotide sequences encoding togaviral glycoproteins, advantageously produce retroviruses pseudotyped with togaviral glycoproteins at a titer of at least

about 1 x 10^3 transforming units (TU)/ml of cell culture supernatant medium. The cells more preferably produce such retroviruses at a titer of at least about 1 x 10^5 TU/ml of supernatant and most preferably at a titer of at least about 1 x 10^6 TU/ml of supernatant.

It is expected that other viruses not specifically mentioned herein having at least two different glycoproteins of similar structure to the glycoproteins in the viral families denoted above may be advantageously used in the present invention.

In another embodiment, the cells include nucleotide sequences encoding glycoproteins from a filovirus. Such filoviruses also exhibit a broad host range. A wide variety of nucleotide sequences that encode filoviral glycoproteins may be used to produce the inventive cells of the present invention. For example, nucleotide sequences encoding glycoproteins from the Marburg and Ebola virus (in the family Filoviridae and, including, for example, Ebola-Zaire and Ebola-Reston) may be introduced into the cells described above to produce a pseudotyped retrovirus. SEQ ID 2 shows the Ebola Zaire glycoprotein-encoding sequence and SEQ ID 3 shows the Marburg virus glycoprotein-encoding sequence. The nucleotide sequences encoding the filoviral glycoproteins may be obtained as described in Sanchez et al. (1993) *Virus Res.* 29 (3):215-240 and Will et al., (1993) *J. Virol.* 67:1203-1210. Moreover, such sequences may be obtained by other methods known to those skilled in the art, as described above for the togaviruses.

Eukaryotic cells described above that include the filoviral nucleotide sequences advantageously produce retroviruses pseudotyped with a filoviral glycoprotein at a titer of at least about 4.5 x 10^4 TU/ml of supernatant. The cells more preferably produce such retroviruses at a titer of at least about 1 x 10^6 TU/ml of supernatant and most preferably at a titer of at least about 1x 10^7 TU/ml of supernatant.

It is expected that other viruses not specifically mentioned above and having glycoproteins of similar structure to the filoviral glycoproteins may be advantageously used in the present invention.

The cells may transiently produce the retrovirus pseudotyped with at least two different viral glycoproteins, such as togaviral glycoproteins, or with a filoviral glycoprotein, but preferably stably produce such retroviruses. In one preferred form of the present invention, the nucleotide sequences encoding either the filoviral glycoproteins or encoding at least two different viral glycoproteins (such as togaviral glycoproteins) in the eukaryotic cells are chromosomally-integrated, so that the cell stably produces the pseudotyped retrovirus. By "stably produce", it is meant that the cells will produce pseudotyped retrovirus indefinitely (i.e., during the life span of the cell). Conversely, by transient production, it is meant that the cells will produce pseudotyped retrovirus for a period of at least about 24 hours, more preferably at least about 48 hours, and most preferably at least about 72 hours.

In a further preferred form of the present invention, the eukaryotic cells described above may include another nucleotide sequence that encodes a desired protein so that they may produce pseudotyped retroviruses having an RNA genome including such desired nucleotide sequences. The protein can be such that it provides a beneficial or therapeutic effect if introduced into an animal. For example, a gene may encode a protein that is needed by an animal, either because the protein is no longer produced, is produced in insufficient quantities to be effective in performing its function, or is mutated such that it either no longer functions or is only partially active for its intended function. The nucleotide sequence may be introduced into the cellular genome in a variety of ways known to the skilled artisan. For example, defective retroviruses (i.e., those which do not have the capability to produce all of the viral proteins necessary for production of a retrovirus having the ability to infect a cell and produce progeny viruses) may be constructed to include such a sequence in their

RNA genome and can then transduce a cell. Alternatively, and as described above, plasmid vectors may be used to introduce the nucleotide sequence, preferably DNA, encoding the desired protein. In either case, the vector typically includes nucleotide sequences necessary for production of the pseudotyped retrovirus. For example, the RNA sequence in the viral genome is flanked on the 5' end by a splice acceptor site and a splice donor site followed by a sequence necessary for packing of the viral genome (such as a psi sequence) and a long terminal repeat (LTR), all as known in the art. The 3' end of the RNA sequence may be flanked on its 3' end with a polypurine tract followed by another LTR, further as known to the skilled artisan. The vectors may include other nucleotide sequences known to the art that are necessary for transduction.

In one preferred form, the desired protein may be one that allows entry of the virus into a cell to be detected. For example, a visually detectable component, or marker, such as one that emits visible wavelengths of light, or that may be reacted with a substrate to produce color of specified wavelengths. For example, such nucleotide sequences include the nucleotide sequence encoding the *Aequorea victoria* green fluorescent protein [GFP; nucleotide sequences listed in Prasher et al., (1992) *Gene* 111:229] and the LacZ gene (produces β-galactosidase), both of which are well known in the art and may be obtained commercially.

A second aspect of the invention provides methods of forming eukaryotic cells for producing pseudotyped retroviruses. The method includes introducing into the cells described above the nucleotide sequences described above, i.e., those encoding the retroviral Gag, Pro and Pol polypeptides, and those encoding either a filoviral glycoprotein or at least two different viral glycoproteins, such as togaviral glycoproteins, into the cell.

The nucleotide sequences may be introduced into the desired cell utilizing a variety of vectors known to the skilled artisan. For example, plasmid vectors, cosmid vectors, and other viral vectors, such as retroviral

vectors, may be used. It is preferred that the nucleotide sequences encoding the Gag, Pro and Pol polypeptides are on a separate vector than the nucleotide sequences encoding the viral glycoproteins.

In one mode of practicing the invention, plasmid vectors are advantageously used to introduce, or transfect, the nucleotide sequences into the selected cell. A wide variety of plasmid vectors may be used, including pTRE, pCMV-Script and pcDNA3, although pcDNA3 is a preferred vector. The *gag, pro* and *pol* nucleotide sequences are preferably on the same plasmid, and, as discussed above, are preferably contiguous to each other. However, the skilled artisan is aware that other spatial configurations of the nucleotide sequences may be utilized when constructing the plasmids. The vector also preferably includes a promoter 5' to, or upstream from, the *gag* nucleotide sequence. The vectors may further include other regulatory elements, such as enhancer sequences, as discussed above.

The nucleotide sequences encoding the viral glycoproteins are preferably on a separate plasmid, or other vector, than the *gag*, *pro* and *pol* nucleotide sequences. The viral glycoprotein encoding sequences, such as the sequences encoding either the filoviral glycoproteins or those encoding at least two different viral glycoproteins (such as togaviral glycoproteins) are also preferably operably linked to a promoter sequence described above. It is also understood that the nucleotide sequences encoding at least two different viral glycoproteins may be arranged on a vector such that the nucleotide sequences encoding one of the glycoproteins are present on one vector and the sequences encoding the other glycoprotein are present on a different vector. It is preferred, however, that such sequences are on the same vector, and preferably contiguous to each other so they will be transcribed utilizing the same promoter. In one preferred form of the invention, the promoter sequence is a cytomegalovirus promoter sequence. Plasmids, or other vectors carrying the nucleotide sequences encoding the viral glycoproteins, may

also include other regulatory elements, such as enhancers, as described above.

The vectors may be introduced into the cells in a variety of ways known to the skilled artisan, for example, discussed in *Current Protocols in Molecular Biology*, John Wiley and Sons, edited by Ausubel et al. (1988) and Maniatis, et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory (1989). For example, vectors may be transfected into a cell by a calcium phosphate precipitation method. Other methods for introduction of the vectors include, for example, electroporation and lipofection.

The nucleotide sequences may be introduced into the cells by a transient transfection procedure such that the proteins encoded by the respective sequences will be produced in a transient fashion as described above. By introducing the MMLV gene sequences and the E_2-E_1 coding region from the Ross River virus (RRV) described above into a cell, we have determined that the cell lines produce pseudotyped retrovirus for a period of about 48 hours. However, it is preferred that the sequences are stably introduced. That is, it is preferred the nucleotide sequences become integrated into chromosomes of the cells into which they are introduced. In this way, the cells will stably produce pseudotyped retrovirus for a longer period of time compared to the transient expression. As used herein, a "stable cell line" or "stable cell" is defined as one that has chromosomally-integrated the nucleotide sequences described above and can produce pseudotyped retrovirus indefinitely (i.e., for the life span of the cell).

Furthermore, in order to form such stable cells, it is necessary to use selectable markers to screen for cells which have chromosomally-integrated the introduced DNA. Accordingly, the plasmid vectors, or other vectors, into which the respective nucleotide sequences are cloned may include such selectable markers.

A wide variety of selectable markers may be used. Typical selectable markers allow growth of only those cells which have been

transfected or transduced and thereby stably produce a desired protein. Examples of selectable markers that may be used include antibiotic resistance genes, including the neomycin gene, the hygromycin phosphotransferase gene and the bleomycin resistance gene which confer resistance to G418, hygromycin and zeocin, respectively. Other selectable markers include, for example, mutant mouse dihydrofolate reductase gene (confers resistance to methotrexate), and the bacterial gpt gene (selects for cells that can grow in a medium containing mycophenolic acid, xanthin and aminopterin). These selectable markers are discussed in *Retroviruses*, Cold Spring Harbor Laboratory Press, p. 444, edited by Coffin, J.M, Hughes, S.H. and Varmus, H.E. (1997).

In many cases, one may wish to quickly visually detect those cells which have taken up a vector and that produce a specified protein from the vector. Visually detectable components, or markers, include the *Aequorea victoria* green fluorescent protein as discussed above. When forming a cell that includes a visually detectable component, or marker, the nucleotide sequences encoding the marker may also be introduced into the cell as described above. For example, the nucleotide sequence encoding the green fluorescent protein may be placed in a recombinant MMLV genome or in a plasmid (to form plasmid MFG.S-GFP) by methods known to the art. For example, plasmid MFG.S-GFP may be formed by including in plasmid MFG [produced by methods known in the art and as exemplified by Ory et al., *PNAS USA*, 93:11400-11406 (1996)] the nucleotide sequence encoding the green fluorescent protein, surrounded by the nucleotide sequences described above, such as LTRs and the psi sequence. Cells that have taken up the vector and express the nucleotide sequences encoding a protein may be identified and separated from cells that do not express the sequences by a fluorescensce-activated cell sorting procedure as known in the art. A visually detectable marker may also be formed from reaction of β-galactosidase (produced by the LacZ gene) with a substrate, such as X-gal.

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Moreover, when growing cells that produce inventive pseudotyped retroviruses, the cells should be grown to no more than about 50% confluency, more preferably no more than about 25% confluency, and the pH of the culture medium should be maintained at about 7 by the frequent changing of culture medium. These conditions are conducive for production of cells that stably produce the pseudotyped retroviruses and should be strictly followed.

In a third aspect of the present invention, pseudotyped retroviruses that include viral glycoproteins (as discussed above) disposed in their lipid bilayer are provided. In one embodiment, at least two different viral glycoproteins are present in the lipid bilayer, such as togaviral glycoproteins. In alternative embodiments the glycoprotein is a filoviral glycoprotein.

In one embodiment, such pseudotyped retroviruses include a core RNA genome that is surrounded by, or enclosed within, a viral capsid. The genome preferably includes a nucleotide sequence encoding a protein selected to be subsequently produced by a cell. The genome further includes other nucleotide sequences for formation of the pseudotyped retrovirus, such as 5' and 3' LTR sequences that are operably linked to the nucleotide sequence encoding the desired protein as described above. Reverse transcriptase and integrase are also enclosed within the capsid, which gives the retrovirus the ability to incorporate a gene encoding a desired protein into a genome of a cell after the retrovirus contacts, or is incubated with, the cell. For example, the pseudotyped retrovirus may be used to incorporate a gene encoding an enzyme in a host cell that is incapable of producing the enzyme, or produces a non-functional enzyme as discussed above. Other sequences known to the art that are useful for transducing genes may also be present in the RNA genome.

The pseudotyped retrovirus may include other proteins, in addition to integrase, that aid its stable integration into the chromosomes of a target

cell. For example, with respect to a lentivirus, the pseudotyped retrovirus may include proteins such as vpr, vif and vpu.

In yet other preferred embodiments, the pseudotyped retrovirus may include a nucleotide sequence encoding a visually detectable component, or marker, such as *Aequorea victoria* green fluorescent protein as discussed above. Such a retrovirus may be advantageously used in a method of determining viral entry into a cell discussed above. Moreover, such a virus is advantageously used in the methods of the present invention to ensure that the pseudotyped retroviruses that are formed are replication incompetent (i.e., do not have all the sequences necessary in their viral genome to produce progeny retroviruses). For example, supernatant isolated from cells transduced by the vectors and contacted with a test cell should not result in localization of the fluorescent protein in the test cell.

In a fourth aspect of the present invention, methods of introducing nucleotide sequences into a cell are provided. In one embodiment, the method includes contacting, or transducing, a cell permissive for either filoviral entry, or entry of a virus having at least two different viral glycoproteins in its lipid bilayer such as a togavirus, with a retrovirus that has been pseudotyped with a filoviral glycoprotein or at least two different viral glycoproteins, such as togaviral glycoproteins, as described above that includes the desired nucleotide sequence in its genome. When the nucleotide sequences encode a desired protein, the cell is selected so that it also preferably allows expression of the selected nucleotide sequence. The level of transduction may be obtained by assaying methods known to the skilled artisan, and include assaying for the protein of interest encoded by the introduced nucleotide sequences or assaying for the presence of the nucleotide sequences. Viruses having at least two different viral glycoproteins in their lipid bilayer have a broad host range. For example, as togaviruses are pantropic (i.e., can invade, or infect, many different cell types with no special affinity for any particular cell type), a wide variety of

permissive cell types well known to the art may be chosen for use in the method, including for example, skin cells, muscle cells, fibroblasts, fat cells and central nervous system cells.

Other viruses having at least two viral glycoproteins in their lipid bilayer include those previously described above. Cells permissive for these viruses are well known to the skilled artisan. Similarly, as filoviruses infect a broad range of cells, a wide variety of cells known to the art that are permissive for filovirus entry may also be selected, including, for example, kidney cells, liver cells, muscle cells and fibroblasts.

In a fifth aspect of the present invention, methods of screening agents effective in blocking viral entry into a cell are provided. The methods allow for direct screening as the viral entry step can be detected in the method. If such agents were tested with a wild type virus, for example, multiple rounds of replication may occur and steps other than viral entry may thus be affected (e.g., such as replication of RNA, production of proteins, etc.). In such a case, one would not know if the agent affects the entry step or some other, indirect step. Thus, the present method allows for direct quantitation of viral entry as compared to remote quantitation.

In one embodiment of the methods of the present invention, a method includes (a) treating a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer that surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer and a nucleotide sequence encoding a marker, preferably a visually detectable marker (or one that is capable of visual detection as described above) that is enclosed within the retroviral capsid, with an agent effective in blocking entry into a cell of the virus having at least two different viral glycoproteins in its lipid bilayer to form a treated pseudotyped retrovirus; (b) treating a cell permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer with the treated pseudotyped retrovirus; and (c) identifying cells having the desired marker. In one embodiment, the retrovirus may have togaviral glycoproteins disposed in its lipid bilayer, and

the cells are permissive for togaviral entry. In alternative embodiments, the retrovirus may have a filoviral glycoprotein, such as a Marburg virus glycoprotein, disposed in its lipid bilayer, wherein the cells that are treated are permissive for Marburg virus entry.

Cells that are advantageously used in a method of screening agents effective in blocking viral entry into a cell are those that are permissive for entry of the specific virus, and will therefore depend on the virus used. Cells permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer are the same as recited in the method of introducing nucleotide sequences into a cell as discussed above. Similarly, cells permissive for Marburg virus entry include those described above used in the method of introducing nucleotide sequences into a cell. If it is not known whether a cell is permissive for viral entry, this can readily be determined by the skilled artisan using routine procedures. One way of determining whether a cell is permissive for viral entry is to transduce the cell with a pseudotyped retrovirus of the present method encoding a marker, and cells that have the marker may be identified by methods known to the art. The marker may be a visually detectable marker, such as the green fluorescent protein or β-galactosidase (i.e., one that gives rise to a visually detectable marker) described above. The selected cell should also allow for expression of the gene products encoded and carried on the viral genome

A wide variety of agents may advantageously be screened in the present invention, including, immunological agents such as monoclonal and/or polyclonal antibodies. For example, monoclonal antibodies or polyclonal antisera against E_2, or other viral glycoproteins, may advantageously be used. Various pharmacological agents may also be screened in the present method in the same way, and include proteins, peptides or various chemical agents.

In one preferred method, the vector, in (a) above, is treated, or incubated with, the agent for a time period sufficient for interaction of the

agent with the viral glycoprotein. Although this time period may vary depending on the nature of the agent and the viral glycoprotein, agents effective in blocking viral entry tend to effectively interact with the glycoprotein in a period of about 10 to about 60 minutes.

In (b), the cell is incubated, or contacted, with the treated pseudotyped retrovirus for a time period sufficient for viral entry. This time period may vary, depending on the specific cell type chosen and the specific viral glycoprotein present in the lipid bilayer of the pseudotyped retrovirus as the skilled artisan knows. However, the time period can typically range from about 1 to about 6 hours, but is typically about 1 to about 2 hours.

Cells having the desired marker may be identified in (c) by observing the presence of the marker. Any of the visually detectable markers previously described above may be utilized in the method. However, a preferred marker is the *Aequorea victoria* green fluorescent protein. Cells into which this marker has been introduced may be identified and separated from cells without the marker (cells not transduced by the retrovirus) by fluorescence-activated cell sorting as described above.

Furthermore, yet another embodiment of a method of screening agents effective in blocking viral entry into a cell includes (1) treating a cell permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer with the agent to form a treated cell; (2) contacting the treated cell with a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer that surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer and a nucleotide sequence encoding a marker, preferably a visually detectable marker (or one that is capable of visual detection as described above), that is enclosed within the retroviral capsid; and (3) identifying cells having the desired marker. As above, the retrovirus may have togaviral glycoproteins disposed in its lipid bilayer, and the cells are permissive for togaviral entry. In alternative embodiments, the retrovirus may have a filoviral glycoprotein,

such as a Marburg virus glycoprotein, disposed in its lipid bilayer, wherein the cells that are treated are permissive for Marburg virus entry. The cells and agents advantageously used in this embodiment are the same as described in the previous embodiment.

In this alternative embodiment, the cells in (1) above are treated, or incubated with, the agent for a time period sufficient for interaction of the agent with the cell to form a treated cell. Although this time period may vary depending on the nature of the agent and the cell, agents effective in blocking viral entry tend to effectively interact with the cell in a period of about 1 hour.

In (2), the treated cell is incubated, or contacted, with the pseudotyped retrovirus for a time period sufficient for viral entry. The time period may vary, depending on the specific cell type chosen and the specific viral glycoprotein in the lipid bilayer of the pseudotyped retrovirus as the skilled artisan knows. However, the time period ranges from about about 1 to about 6 hours, but is typically about 1 to about 2 hours.

Cells having the desired marker may be identified in (3) by the same method as described in (c) of the previous embodiment.

In a sixth aspect of the present invention, kits for forming inventive pseudotyped retroviruses are provided. The kits include a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one embodiment of the invention, the fourth nucleotide sequence encodes at least two different viral glycoproteins, such as togaviral glycoproteins and preferably alphaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a filoviral glycoprotein, preferably a Marburg virus glycoprotein. The sequences and methods of obtaining such sequences are discussed above. In general, the kits include sterile packaging which secures the various kit components in spaced relation from one another

sufficient to prevent breakage of the components during handling of the kit. For example, it is a common practice to utilize molded plastic articles having multiple compartments or areas for holding the kit components in spaced relation.

The inventive pseudotyped retrovirus are further useful in methods of identifying cell surface receptors that allow viral entry. In one embodiment, an inventive pseudotyped retrovirus may be employed in a method that identifies cell surface receptors for a virus having at least two different viral glycoproteins disposed in its lipid bilayer. The method includes (a) constructing a cDNA library from a first cell that is permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer; (b) transfecting a second cell with a cDNA-carrying vector wherein the second cell is non-permissive or semi-permissive for entry of a pseudotyped retrovirus that includes a retroviral capsid, a lipid bilayer wherein the lipid bilayer surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer and a nucleotide sequence encoding a desired marker wherein the nucleotide sequence is enclosed within the retroviral capsid; (c) transducing the second cell with the pseudotyped retrovirus; (d) identifying cells having the marker; and (e) identifying the cDNA insert in the transduced cell. In alternative embodiments, the cDNA library is constructed from a first cell permissive for entry of a Marburg virus and the second cell is transduced with a retrovirus pseudotyped with the Marburg virus glycoprotein.

In a preferred method, the first cell is permissive for togaviral entry, further preferably alphaviral entry, and the second cell is transduced with a retrovirus pseudotyped with togaviral glycoproteins, preferably alphaviral glycoproteins.

In (a), a cDNA library may be constructed by methods well known to the skilled artisan as described in *Current Protocols in Molecular Biology*, John Wiley and Sons, edited by Ausubel et al. (1988). For example, mRNA may be isolated from the first cell by breaking the cell membrane and

extracting and purifying the mRNA by known methods. The mRNA may be used as a template to form cDNA,which may then be cloned into various vectors as described above, such as plasmid vectors, by use of various restriction enzymes and DNA ligase as known in the art. Bacterial cells, or other similar cells, may be transfected with the expression vectors to form the cDNA library.

The first cell may be chosen from the cells permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer, such as an alphavirus, or a virus having a filoviral glycoprotein disposed in its lipid bilayer, such as a Marburg virus glycoprotein, or other filovirus glycoprotein as described above.

In (c), the second cell may be transduced with a pseudotyped retrovirus having a nucleotide sequence encoding a desired marker as described above in the embodiment described above of the method for screening agents effective in blocking viral entry into a cell and in (d), the transduced cells may be identified by methods discussed above, such as fluorescence activated cell sorting.

The second cell may be selected from non-permissive cells, preferably mammalian, known in the art. For example, in the case of the pseudotyped retrovirus that includes at least two viral glycoproteins disposed in its lipid bilayer, such as those from the Ross River virus, non-permissive cells include chicken embryo fibroblasts. One skilled in the art may also determine what other cells are non-permissive for alphaviruses, such as the Ross River virus, and the filoviruses, such as Marburg or Ebola virus, by the methods described herein as well as other methods known to the art.

The cDNA insert in the transduced eukaryotic cell may be identified and recovered by known methods, including amplifying known sequences in the cDNA-containing plasmids by PCR.

Reference will now be made to specific examples illustrating the compositions and methods above. It is to be understood that the examples

are provided to illustrate preferred embodiments and that no limitation to the scope of the invention is intended thereby.

EXAMPLE 1

Cells and Cell Culture

E86nlslacZ cells, Baby Hamster Kidney (BHK) cells, and mouse NIH3T3 fibroblasts were grown in Dulbecco's Modified Eagle Media (D-MEM, Sigma) with 10% Calf Serum (Gibco-BRL), 0.1 mg/ml streptomycin (Sigma) and 10 U/ml penicillin (Sigma)(D-MEM CS/PS). E86nlslacZ cells are NIH 3T3 cells that express MMLV capsid proteins, produced as known in the art and as described in Taylor, G.M. and Sanders, D.A. (1999) *Mol. Biol. of the Cell* (1999), in press, were constructed by stably transfecting GP+E86 cells of Markowitz et al. (1988) *J. of Virol.* 62:1120-1124 with MFG.S-nlslacZ. MFG.S-nlsLacZ is a retroviral vector encoding a nuclear localized β-galactosidase activity, produced as known in the art and as described in Ory, et al. (1996) *PNAS USA* 93:11400-11406.

Human HeLa, ΦNX cells, gpGFP and gpnlslacZ cells were grown in D-MEM FBS/PS). ΦNX packaging cells are second generation human embryonic kidney 293T cells transfected with MMLV *gag* and *pol* genes as described in Grignani et al. (1998) *Cancer Res.*, 58:14-19 and Pear et al., (1993) *PNAS USA*, 90:8392-8396. gpGFP cells are obtained by transfecting ΦNX cells with retroviral vector MFG.S-GFP-S65T, a retroviral vector encoding the *Aequorea victoria* green fluorescent protein S65T mutant as described in Taylor, G.M. and Sanders, D.A. (1999) *Mol. Biol. of the Cell* (1999), in press. gpGFP cells therefore produce envelope-deficient replication- incompetent MMLV particles carrying MFG.S-GFP-S65T. gpnlslacZ cells were developed in our laboratory by cotransfecting MFG.S-nlsLacZ and pJ6Ωpuro [constructed as described in Morgenstern and Land (1990), *Nucleic Acids Res.*, 18:1068] into ΦNX cells, growing transfected cells in D-MEM FBS/PS supplemented with 2 μg/ml puromycin (Sigma) and antibiotic-resistant colonies were isolated and screened for the

production of high-titer replication-incompetent virus resulting from transient transfection with penv1min, a vector that encodes the wild type MMLV envelope protein [as described in Taylor, G.M. and Sanders, D.A. (1999) *Mol. Biol. of the Cell* (1999), in press].

VSV-G pseudotyped retrovirus-producing 293GPGnlslacZ cells, constructed as described in Ory, et al. (1996) *PNAS USA* 93:11400-11406, were grown in D-MEM FBS/PS supplemented with 2 µg/ml puromycin and 1 µg/ml tetracycline (Sigma). As expression of the VSV-G protein in these cells is repressed by the presence of tetracycline in the medium, forty-eight hours before collection of pseudotyped virus the medium in which the 293GPGnlslacZ cells were grown was replaced with D-MEM FBS/PS.

All cells were grown at 37°C and under 5% CO_2. Moreover, the cells were grown at a density of no more than about 50% confluency and the medium was changed at intervals sufficient to maintain the pH of the medium at about 7.

EXAMPLE 2

Generation of Cell Lines Transiently Producing RRV-MMLV Pseudotyped Retrovirus

RRV Glycoprotein Expression Plasmid Construction

The region encoding the Ross River virus envelope glycoproteins was amplified from pRR64, a plasmid which contains the full-length Ross River viral genome [full-length sequence described in Faragher et al., (1988), *Virology* 163:509-526] as described in Kuhn et al. (1991), *Virology* 182:430-41, by the polymerase chain reaction using Pfu polymerase and two primers complementary to the viral genome at nucleotides 8375-8386 (5'-CGGGATCCACCATGTCTGCCGCGCT-3') and 11312-11330 (5'-CGCTCTAGATTACCGACGCATTGTTATG-3') [the amplified sequences from plasmid pRR64 are shown in SEQ ID 1, beginning at nucleotide 3, and an additional "at" sequence (nucleotides 1 and 2 of SEQ ID 1) was

added at the 5' end of the pRR64 sequence]. The amplified fragment, which contained the RRV E_3-E_2-6K-E_1 coding region, was digested with the restriction endonucleases Bam HI and Xbal and ligated into BamHI and Xbal sites of pBacPac, a Baculovirus expression vector available from Clontech. The resulting plasmid was digested with BamHI and Xbal, and the fragment containing the RRV E_3-E_2-6K-E_1 coding region was ligated into the BamHI and Xbal sites in the pcDNA3 mammalian expression vector available from Invitrogen. The resulting plasmid was designated pRRV-E_2-E_1. SEQ ID 1 also shows the amino acid sequence of the E_3-E_2-6K-E_1 polypeptide.

Transient Transfection Procedure

In preparation for transfection, 0.5 x 10^6 ΦNX cells, or gpnlslacZ cells, were washed with PBS (137 mM NaCl, 27 mM KCl, 4.3 mM Na_2HPO4, 1.47 mM K_2HPO4, pH 7.4) prior to incubation with 2 ml Opti-MEM (Gibco-BRL) for 30 minutes at 37°C in a 5% CO_2 atmosphere. 2 µg of pRRV-E_2-E_1 and 2 µg of MFG.S-GFP-S65T was incubated with 300 ml Opti-MEM and 24 ml lipofectAMINE™ (Gibco-BRL) for 30 minutes at room temperature prior to dilution with 2.4 ml Opti-MEM. The resulting mixture was incubated with the cells for seven hours at 37°C in a 5% CO_2 atmosphere. Medium was replaced with DMEM FBS/PS for a further 48-hour incubation at 37°C in a 5% CO_2 atmosphere before collection of the supernatant medium for analysis of the transduction capacity of and level of glycoprotein incorporation into viral particles. When the gpnlslacZ cells were transfected, a similar protocol was followed except that the transfected DNA consisted solely of 4 µg of pRRV-E_2-E_1.

Transduction by Recombinant Retroviruses

HeLa, BHK and NIH 3T3 cells were transduced by the following method. Supernatant medium from recombinant-virus-producing cells was filtered through a 0.45 µm filter, mixed with hexadimethrine bromide

(Sigma) (final concentration 8 μg/ml) and incubated with cells for five hours at 37°C in a 5% CO_2 atmosphere. The recombinant virus-containing medium was then replaced with D-MEM CS/PS medium. Cells transduced with MFG.S-GFP-S65T were washed 48 hours after infection with PBS, then lifted from the plate with PBS containing 1 mM EDTA. The cells were then analyzed with a Coulter XL-MCL Flow Cytometer using a 525 nm band-pass and a 488 nm air-cooled argon laser. The level of glycoprotein incorporation into viral particles was determined by Western blotting as described in Example 4.

Analysis

Cell have been constructed that produce infectious pseudotyped virus containing the glycoproteins from the Ross River virus. The titer of virus was found to be 1×10^3 TU/ml supernatant. The cells were able to produce the pseudotyped retrovirus for a period of 48 hours. As MMLV only infects mouse cells such as NIH 3T3 and the Ross River virus glycoprotein-pseudotyped retrovirus is able to infect NIH 3T3 cells, as well as BHK (hamster) and HeLa (human) cells, it has clearly been demonstrated that the host range of these retroviruses is increased by incorporation of the Ross River glycoproteins in the virus.

This example also shows that at least two different viral glycoproteins, each having a different membrane-spanning domain, can be incorporated into a retroviral particle (i.e., a retrovirus).

EXAMPLE 3

Generation of Stable Cell Lines Producing RRV-MMLV Pseudotyp d Retrovirus

Stable Transfection Procedure

0.5×10^6 ΦNX or gpnlslacZ cells were transfected following the protocol in Example 2, except that the DNA that was transferred was only 8 µg of pRRV-E_2-E_1 and 0.4µg of plasmid pJ6Ωpuro coding for puromycin resistance [Morgenstern and Land, *Nucleic Acids Res.* 18, 1068 (1990)] and the DNA mixture contained 48 µl lipofectAMINE™ and 600 µl Optim-MEM. Selection with medium containing 2 µg/mL puromycin began at 48 hours post-transformation. Clonal colonies of cells were isolated after two weeks of selection. The resulting cell lines derived from the ΦNX cells were designated SafeRR and the resulting cell lines derived from the gpnlslacZ cells were designated SafeRRnlslacZ.

Titer was measured by infection of NIH3T3 cells as described below. Infection occurred in the presence of 8 µg/ml polybrene and infectious supernatant was changed to media without polybrene 5 hours post-infection.

Transduction by Recombinant Retroviruses

The same protocol of Example 2 was followed, except that forty-eight hours after the infection, the cells transduced with virus bearing MFG.S-nlslacZ were fixed with 0.5% glutaraldehyde (Sigma) and then incubated with 1 mg/ml of the β -galactosidase detection reagent 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Fisher) in a staining buffer (1 mM $MgCl_2$, 50 mM $K_3Fe(CN)_6$ and 50 mM $K_4Fe(CN)_6$) for 3 hours prior to the determination of the proportion of blue cells as provided in Sanes, et al. (1986), *Embo J.*, 5:3133-3142.

Analysis

Cells that permanently produce the above pseudotyped retroviruses have been constructed. SafeRR cells were found to produce pseudotyped

retrovirus at a titer of 1 x 10^3 TU/ml supernatant. SafeRR-nlslacZ cells were found to produce pseudotyped retrovirus at a titer of about 1 x 10^5 TU/ml supernatant. SafeRR cells may be advantageous in introducing desired nucleotide sequences into a cell. Another advantage is that the expression of the Ross River virus glycoproteins are not toxic to the cells.

EXAMPLE 4

Immunodetection of Incorporation of RRV-E_2 into Pseudotyped Retrovirus Produced by SafeRR-nslacZ cells

This example demonstrates that the recombinant retrovirus contains the Ross River glycoproteins.

Supernatant medium from a 10 cm tissue-culture dish of confluent SafeRRnlslacZ cells (described in Example 3), or precursor gpnlslacZ cells (described in Example 1), was passed through a 0.45 µm filter and spun through a 30% sucrose cushion at 25K rpm for 2.5 hours in a Beckman 50.2 titanium rotor. Material collected through the centrifugation was suspended in SDS-PAGE buffer (0.05% bromophenol blue, 0.0625 M Tris-HCl pH 6.8, 1% SDS, 10% glycerol). Cell lysates were prepared by washing cells with 10 ml PBS followed by 2 ml cell lysis buffer (50 mM Tris-HCL, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100). Aliquots of total lysed material were mixed with SDS-PAGE buffer and analyzed electrophoretically. PAGE-separated proteins were transferred to nitrocellulose membranes at 44 mA for 2 hours in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were blocked with 5% powdered milk in washing solution (20 mM Tris-HCl, pH7.6, 137 mM NaCl, 0.1% Tween-20). Blocked membranes were reacted with pAbE2 (anti-Ross River E_2 rabbit polyclonal antiserum; provided by Richard Kuhn and produced by methods known to the art) at a 1:5000 dilution for two hours and goat anti-rabbit Horseradish Peroxidase (HRP)-linked secondary antibody (Chemicon, 1 mg/ml) at a 1:5000 dilution for thirty minutes.

Western blots were visualized with Enhanced Chemiluminescent Reagents (Amersham Pharmacia Biotech) by methods known in the art.

Analysis

In order to clarify that E_2-E_1 were incorporated into the MMLV particles and could be mediating the infection observed in Example 3, both virus producing cells and infectious supernatants were analyzed by SDS-PAGE and Western blotting with a polyclonal E_2 antiserum.

As seen in FIG. 1, a 50kDa and a 60kDa immunoreactive protein were present in a lysate of SafeRRnlslacZ (express RRV E_2-E_1 pseudotyped MMLV). These are appropriate masses for E_2 and unprocessed E_2-E_3. Western analysis of virus collected from infectious supernatant revealed only the fully processed 50 kDa protein.

EXAMPLE 5

Formation of Syncytia in Stable SafeRR-nlslacZ Cell Lines at Acidic pH

This example shows that SafeRR-nlslacZ cell lines are capable of forming syncytia at acidic pH, implying that entry of alphavirus into cells is dependent on the low pH environment normally found in endosomes.

SafeRR-nlslacZ or ΦNX cells, obtained as described in Examples 3 and 1, respectively, were grown to near confluence, washed with PBS and treated with fusion buffer [PBS containing 10 mM 2-(N-morpholino)ethane sulfonic acid and 10 mM HEPES adjusted to pH 5.5] for one minute. The low pH solution was replaced with D-MEM FBS/PS, then cells were incubated in a CO_2 incubator at 37°C, and the cells were stained with Giemsa solution 5 hours after treatment and photographed.

Analysis

As seen in FIG. 2A, syncytia are detectable. No syncytia were observed in the treated ΦNX cells that are shown in FIG. 2B. It is seen in

FIG. 2C that syncytia are also not detected when the SafeRR-nlslacZ cells are incubated in pH 7 fusion buffer. These results, indicating that Ross River virus glycoprotein-promoted membrane fusion is triggered by an acidic medium, are consistent with the data obtained by other laboratories that indicate the entry of alphavirus is dependent upon the low pH environment normally found in endosomes [other data discussed in Strauss and Strauss, (1994) *Microbiol. Rev*, 58:491-562].

EXAMPLE 6

Effect of Lysosomotropic Weak Bases on Infection By RRV-MMLV Pseudotyped Retrovirus

This example shows that the RRV-MMLV pseudotyped retrovirus enters cells through an endocytic pathway.

NIH 3T3 cells were pretreated for one hour with various concentrations of ammonium chloride or chloroquine in PBS as seen in FIG. 3. Medium containing 1.5 x 10^5 TU/ml of supernatant of either wild type MMLV, VSV-G pseudotyped retrovirus or Ross-River E_2-E_1 pseudotyped retrovirus (produced by SafeRRnlslacZ cells) containing various concentrations of bases (as seen in FIG. 3) as well as 8 µg/ml polybrene was incubated with the cells in a CO_2 incubator at 37°C. The virus-containing medium was replaced with D-MEM CS/PS 6 hours after infection. The cells were stained with a β-galactosidase detection reagent (X-gal) at 48 hours post infection, and blue cells were counted. The results are shown in FIG. 3.

Analysis

Ammonium chloride and chloroquine inhibit the acidification of endosomes and inhibit cellular entry of viruses that are taken up by endocytosis and that require exposure to low pH for virus-cell membrane fusion to occur as reported in Marsh and Helenius, *Adv. Virus Res.* (1989), 36:107-151. MMLV entry is known not to involve low pH-induced virus-cell

membrane fusion and infection by VSV-G pseudotyped retrovirus is known to involve low pH-induced virus-cell membrane fusion. These retroviruses therefore served as controls. The results show that chloroquine only partially affects wild type MMLV entry as seen in FIG. 3A, and that both chloroquine and ammonium chloride inhibit VSV-G pseudotyped retrovirus entry. It can therefore be concluded that the dramatic inhibition of transduction by Ross River glycoprotein-pseudotyped viruses in the presence of ammonium chloride and chloroquine is a direct effect upon entry, as all of the macromolecules required for the other necessary processes (viral uncoating, reverse transcription, integration, etc.) are identical with those contained in the relatively uninhibited MMLV-Env-bearing viruses. This example illustrates one of the advantages of the inventive pseudotype system of the present invention; the effects of an experimental manipulation on viral entry into a cell may be specifically investigated independent of any effects on other steps in replication.

EXAMPLE 7

Neutralization of MMLV Pseudotyped with RRV E_2-E_1 Coding Region

This example shows that retroviruses pseudotyped with the Ross River virus E2-E1 are inhibited from entering a cell when pre-incubated with antibodies against E2.

Supernatants from SafeRR-nlslacZ or wild type MMLVnlsLacZ (MMLV that includes RNA encoding β-galactosidase and the *env* gene proteins) producing cells were incubated with dilutions of Ross River virus monoclonal 10C9 [produced as described in Smith, (1995) *PNAS USA* 92:10648-10652] in ascites fluid or dilutions of Ross River virus polyclonal (pAbE2) antiserum (provided by Richard Kuhn and produced by methods known to the art) prior to infection of NIH3T3 cells. No significant inhibition of infectivity was observed in wild type MMLVnlsLacZ while a 60% inhibition of infectivity of RRV-MMLVnlsLacZ was observed at a 1:500

dilution of polyclonal antiserum. Inhibition was most significant with monoclonal 10C9, which binds to the cell receptor binding region on RRV E_2 (Smith et al., *Proc Natl Acad Sci USA* 92, 10648-10652 (1995)). For example, a 70% inhibition of infectivity was observed in supernatant from SafeRR-nlslacZ cells with a 1:500 dilution of ascites fluid containing monoclonal 10C9.

EXAMPLE 8

Generation of Cell Lines Transiently Producing Ebola-MMLV Pseudotyped Retrovirus Including Nucleotide Sequences Encoding GFP in its Genome

This example shows production of cell lines that transiently produce MMLV pseudotyped with Ebola-Zaire glycoprotein.

pEZGP1 was produced by cloning into the polylinker of plasmid pcDNA3 nucleotide sequences corresponding to nucleotides 6029-8253 [sequences 6029-8253, corresponding to nucleotides 132-2354 described in Genbank as Accession Number U23187, are shown in SEQ ID 2 from the Ebola Zaire virus genome, with the exception that an additional "a" has been inserted between nucleotides 1027 and 1028 in SEQ ID 2 compared to the Genbank sequence] from the complete Ebola Zaire genome [described in Sanchez, et al., (1993) *Virus Res.* 29(3):215-240] obtained by digestion of the MP1153 plasmid provided by Dr. Anthony Sanchez with Eco RI and HindIII. SEQ ID 2 also shows the amino acid sequence of the Ebola Zaire glycoprotein.

gpGFP cells were transiently transfected with pEZGP1 using lipofectAMINE™ (Gibco, BRL) and Opti-MEM media (Gibco, BRL). The gpGFP cells were plated at 5x 10^5 cells/60 mm plate 24 hours prior to transfection. The cells were washed and incubated for 30 minutes at 37°C with 2 ml of Opti-MEM media. The DNA-LipofectAMINE™-Opti-MEM mixture (4µg DNA, 24 µl lipofectAMINE™, and 300 µl Opti-MEM media)

was incubated for 30 minutes at 25°C. After the 30 minute incubations, 2.4 ml of Opti-MEM media was added to the DNA-lipofectAMINE™ mixture. The resulting solution was layered onto the gpGFP cells. Eight hours later, the transfection mixture was removed and the cells were incubated with DMEM FBS/PS for 40 hours. The supernatant medium was filtered through a 0.45 µm filter and then incubated with 1 x 10^6 NIH 3T3 cells in the presence of 8 µg/ml polybrene for 4 hours. The recombinant-virus-containing medium was then replaced with D-MEM CS/PS. Forty-eight hours later the cells were removed from the plate, suspended in 1xPBS containing 1 mM EDTA, and analyzed by flow cytometry with a Coulter XL-MCL Flow Cytometer, using a 525 nm band-pass filter and a 488 nm air-cooled argon laser.

Analysis

Cell have been constructed that produce infectious pseudotyped virus containing glycoproteins from the Ebola Zaire virus. The titer of virus was found to be 4.5 x 10^4 TU/ml of supernatant. The cells were able to produce the pseudotyped retrovirus for a period of about 24 hours.

EXAMPLE 9
Generation of Stable Cell Lines Producing Ebola-MMLV Pseudotyped Retrovirus

gpGFP cells were stably transfected with pEZGP1. gpGFP cells were plated at 5x 10^5 cells/60 mm plate 24 hours prior to transfection. The cells were washed and incubated for 30 minutes at 37°C with 2 ml of Opti-MEM media. The DNA-LipofectAMINE™-Opti-MEM mixture (8 µg of mutant DNA, 0.4µg of pJ6Ωbleo, 48 µl lipofectAMINE™, and 300 µl Opti-MEM media) was incubated for 30 minutes at 25°C. After the 30 minute incubations, 2.4 ml of Opti-MEM media was added to the DNA-LipofectAMINE™ mixture. The resulting solution was layered onto the gpGFP cells. Eight hours later the transfection mixture was removed and

the cells were incubated with DMEM FBS/PS for 40 hours before transferring the cells to 10 cm plates at two different dilutions (1/10 and 1/100). The following day, the media was changed to D-MEM FBS/PS containing 200 µg/ml of Zeocin. Colonies appeared after two weeks and were picked for screening by an infectivity assay described below. The cell lines so produced were labeled "SafeEbola-GFP".

The supernatant medium from the cells was filtered through a 0.45 µm filter and then incubated with 1 x 10^6 NIH 3T3 cells in the presence of 8 µg/ml polybrene for 4 hours. The recombinant-virus-containing medium was then replaced with D-MEM CS/PS. Forty-eight hours later the cells were removed from the plate, suspended in 1xPBS containing 1 mM EDTA, and analyzed by flow cytometry with a Coulter XL-MCL Flow Cytometer, using a 525 nm band-pass filter and a 488 nm air-cooled argon laser.

Stable cell lines that produce pseudotyped retrovirus not containing specific nucleotide sequences such as those encoding the green fluorescent protein were produced in the same manner, except the parent cell line to the gpGFP cells were used instead (i.e., ΦNX cells, human embryonic kidney cells transfected only with MMLV *gag* and *pol* nucleotide sequences). These cell lines were labeled "SafeEbola".

As seen in FIG. 4, lower panel B, cells (45.8% as determined by fluorescence activated cell sorting) transduced with pseudotyped retroviruses produced from SafeEbola-GFP cells exhibited detectable green fluorescence.

Analysis

Cell lines that stably produce MMLV virus pseudotyped with Ebola Zaire glycoprotein have been produced. The cells indefinitely produce the pseudotyped retrovirus. The glycoprotein used to form the pseudotyped retrovirus is not toxic. The cells require diligence in care (i.e., changing the media every two days) so that the pH does not drop and syncytia formation does not occur.

EXAMPLE 10

Formation of Syncytia in Stable SafeEBola-GFP Cell Lines at Acidic pH

This example shows that SafeEbola-GFP cell lines are capable of forming syncytia at acidic pH.

5 x 10^5 SafeEbola-GFP cells or ΦNX cells, obtained as described in Examples 10 and 1, respectively, were plated on 60 mm tissue-culture dishes, grown to near confluence, washed with PBS and treated with fusion buffer [PBS containing 10 mM 2-(N-morpholino)ethane sulfonic acid and 10 mM HEPES adjusted to pH 5.5] for one minute. The low pH solution was replaced with D-MEM FBS/PS, incubated in a CO_2 incubator at 37°C, and the cells were stained with Giemsa solution 5 hours after treatment and photographed. As seen in FIG. 5A, the SafeEbola-GFP cell lines form syncytia at acidic pH, whereas no such syncytia are formed in ΦNX cells as seen in FIG. 5B.

EXAMPLE 11

Generation of Cell Lines Transiently Producing Marburg Virus Glycoprotein Pseudotyped Retrovirus

Marburg Glycoprotein Expression Plasmid

Marburg plasmid pMBGP1 was constructed from a plasmid from Hans-Dieter Klenk (Marburg, Germany). To construct this plasmid, the nucleotides 5931-8033 from the Marburg virus genome [the genomic nucleotide sequence HK Klenk, as delineated in Will et al. (1993), *J. Virol.* 67:1203-1210 and as seen in Genbank Accession Number Z12132 shown in SEQ ID 3] were cloned into the pSP72 plasmid (from Promega) under the control of the T7 promoter using SalI. The XhoI and Eco RI fragment of this plasmid was cloned into the XhoI and Eco RI polylinker sites of the

mammalian expression vector pcDNA3. SEQ ID 3 also shows the amino acid sequence of the Marburg virus glycoprotein.

Transient Transfection Procedure

The transient transfection protocol was identical to that recited in Example 8 (Ebola-glycoprotein transfection protocol), with the exception that, instead of pEZGP1, 4 µg of pMBGP1 was used.

Analysis

It has been shown that cell lines may be constructed that produce MMLV that is pseudotyped with the Marburg virus glycoprotein. The cell lines were found to produce the pseudotyped retroviruses at a titer of about 1.4×10^3 TU/ml of supernatant. The cells were able to produce the virus for a period of about 24 hours. In data not shown, it was found that NIH 3T3, BHK and HeLa cells can be efficiently transduced by this inventive pseudotyped retrovirus. This demonstrates the expanded host range of the pseudotyped retroviruses, which allows these pseudotyped retroviruses to be advantageously used to introduce desired nucleotide sequences into target cells.

EXAMPLE 12

Generation of Cell Lines Stably Producing Marburg Virus Glycoprotein Pseudotyped Retrovirus

Stable Transfection Procedure

The stable transfection protocol was identical to that recited in Example 9 (Ebola-glycoprotein transfection protocol), with the exception that 4 µg of pMBGP1 (described in Example 11) was used.

Analysis

It has been shown that cell lines may be constructed that stably, and thus indefinitely, produce MMLV that is pseudotyped with the Marburg virus glycoprotein. The cell lines were found to produce the pseudotyped retroviruses at a titer of about 1.9×10^3 TU/ml of supernatant. The glycoprotein incorporated into the lipid bilayer of the pseudotyped retroviruses is not toxic. Moreover, the cells require diligence in care (i.e., changing of the media every two days) so that the pH does not drop and syncytia formation does not occur.

While the invention has been illustrated and described in detail in the drawings and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiment has been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected. In addition, all references cited herein are indicative of the level of skill in the art and are hereby incorporated by reference in their entirety.

CLAIMS

What is claimed is:

1. A eukaryotic cell, comprising:

(a) a first nucleotide sequence encoding a retroviral Gag polypeptide;

(b) a second nucleotide sequence encoding a retroviral Pro polypeptide;

(c) a third nucleotide sequence encoding a retroviral Pol polypeptide; and

(d) a fourth nucleotide sequence encoding at least two different viral glycoproteins.

2. The cell of claim 1, wherein said cell further comprises a fifth nucleotide sequence having a 5' and a 3' end, said fifth nucleotide sequence encoding a desired protein, said fifth nucleotide sequence operably linked at said 5' end to a first retroviral long terminal repeat sequence and operably linked at said 3' end to a second retroviral long terminal repeat sequence.

3. The cell of claim 2, wherein said desired protein is a marker.

4. The cell of claim 3, wherein said marker is a fluorescent protein.

5. The cell of claim 1, wherein said two different viral glycoproteins are togaviral glycoproteins.

6. The cell of claim 5, wherein said togaviral glycoproteins are alphaviral glycoproteins.

7. The cell of claim 6, wherein said alphaviral glycoprotein is a Ross River alphaviral glycoprotein.

8. The cell of claim 1, wherein said eukaryotic cell is a mammalian cell.

9. The cell of claim 8, whererin said mammalian cell is a human cell.

10. The cell of claim 1, wherein said retroviral Gag, Pol and Pro polypeptides are comprised of Moloney murine leukemia Gag, Pro and Pol polypeptides.

11. The cell of claim 1, wherein said cell produces a pseudotyped retrovirus having a lipid bilayer, said viral glycoproteins disposed in said lipid bilayer.

12. The cell of claim 1, wherein said first, second, third and fourth nucleotide sequences are chromosomally-integrated.

13. A eukaryotic cell, comprising:

(a) a first nucleotide sequence encoding a retroviral Gag polypeptide;

(b) a second nucleotide sequence encoding a retroviral Pro polypeptide;

(c) a third nucleotide sequence encoding a retroviral Pol polypeptide; and

(d) a fourth nucleotide sequence encoding a filoviral glycoprotein, said first, second, third and fourth nucleotide sequences being chromosomally-integrated, said cell stably producing pseudotyped retroviruses.

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14. The cell of claim 13, wherein said cell further comprises a fifth nucleotide sequence having a 5' end and a 3' end, said fifth nucleotide sequence encoding a desired protein, said fifth nucleotide sequence operably linked at said 5' end to a first retroviral long terminal repeat sequence and operably linked at said 3' end to a second retroviral long terminal repeat sequence.

15. The cell of claim 13, wherein said filoviral glycoprotein is selected from the group consisting of Marburg virus glycoprotein and Ebola virus glycoprotein.

16. The cell of claim 13, wherein said retroviral Gag, Pro and Pol polypeptides are comprised of Moloney murine leukemia virus Gag, Pro and Pol polypeptides.

17. The cell of claim 13, wherein said cell produces pseudotyped retrovirus at a titer of at least about 4.5×10^4 transforming units/ml of supernatant.

18. A eukaryotic cell, comprising:

(a) a first nucleotide sequence encoding a retroviral Gag polypeptide;

(b) a second nucleotide sequence encoding a retroviral Pro polypeptide;

(c) a third nucleotide sequence encoding a retroviral Pol polypeptide; and

(d) a fourth nucleotide sequence encoding a Marburg virus glycoprotein.

19. A method of forming a eukaryotic cell for producing pseudotyped retroviruses, said method comprising:

transfecting a eukaryotic cell with a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least two different viral glycoproteins.

20. The method of claim 19, wherein said first, second and third nucleotide sequences are operably linked to a promoter sequence.

21. The method of claim 19, wherein said viral glycoproteins are togaviral glycoproteins.

22. The method of claim 21, wherein said togaviral glycoproteins are alphaviral glycoproteins.

23. The method of claim 22, wherein said alphaviral glycoproteins are Ross River alphaviral glycoproteins.

24. The method of claim 19, wherein said first, second, third and fourth nucleotide sequences are chromosomally-integrated.

25. The method of claim 19, wherein said cell further comprises a fifth nucleotide sequence having a 5' end and a 3' end, said fifth nucleotide sequence encoding a desired protein, said fifth nucleotide sequence operably linked at said 5' end to a first retroviral long terminal repeat sequence and operably linked at said 3' end to a second retroviral long terminal repeat sequence.

26. A method of forming a eukaryotic cell for producing pseudotyped retroviruses, said method comprising:

(a) transfecting a eukaryotic cell with a vector including a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide and a third nucleotide sequence encoding a retroviral Pol polypeptide, said first, second and third nucleotide sequences operably linked to a first promoter sequence; and

(b) transfecting said cell with a fourth nucleotide sequence encoding at least two viral glycoproteins, said fourth nucleotide sequence operably linked to a second promoter sequence.

27. The method of claim 26, said method further comprising transfecting said cell with a vector including a fifth nucleotide sequence having a 5' and a 3' end, said fifth nucleotide sequence encoding a desired protein, said fifth nucleotide sequence operably linked at said 5' end to a first retroviral long terminal repeat sequence and operably linked at said 3' end to a second retroviral long terminal repeat sequence.

28. The method of claim 26, wherein said desired protein is a marker.

29. The method of claim 26, wherein said first, second, third and fourth nucleotide sequences are chromosomally-integrated.

30. A method of forming a eukaryotic cell for producing pseudotyped retroviruses, said method comprising:

(a) transfecting a eukaryotic cell with a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding a

filoviral glycoprotein, said first, second, third and fourth nucleotide sequences being chromosomally-integrated, said cell stably producing pseudotyped retroviruses.

31. The method of claim 30, wherein said filoviral glycoprotein is selected from the group consisting of Ebola virus glycoprotein and Marburg virus glycoprotein.

32. A method of forming a eukaryotic cell for producing pseudotyped retroviruses, said method comprising:

transfecting a eukaryotic cell with a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding a Marburg virus glycoprotein.

33. A pseudotyped retrovirus, comprising:

(a) a retroviral capsid;

(b) a lipid bilayer; said lipid bilayer surrounding said retroviral capsid; and

(c) at least two different viral glycoproteins disposed in said lipid bilayer.

34. The retrovirus of claim 33, said retrovirus further comprising a nucleotide sequence encoding a desired protein, said nucleotide sequence enclosed within said retroviral capsid.

35. The retrovirus of claim 33, wherein said viral glycoproteins are togaviral glycoproteins.

36. The retrovirus of claim 35, wherein said togaviral glycoproteins are alphaviral glycoproteins.

37. The retrovirus of claim 36, wherein said alphaviral glycoproteins are Ross River alphaviral glycoproteins.

38. The retrovirus of claim 33, wherein said retroviral capsid is comprised of a Moloney murine leukemia virus capsid.

39. A pseudotyped retrovirus, comprising:

(a) a retroviral capsid;

(b) a lipid bilayer; said lipid bilayer surrounding said retroviral capsid; and

(c) a Marburg virus glycoprotein disposed in said lipid bilayer.

40. A method of introducing a nucleotide sequence into a cell, said method comprising:

transducing a cell permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer with a pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer; said lipid bilayer surrounding said retroviral capsid;

at least two different viral glycoproteins disposed in said lipid bilayer; and

a desired ribonucleotide sequence.

41. The method of claim 40, wherein said retroviral capsid is a Moloney murine leukemia virus capsid.

42. The method of claim 40, wherein said virus having at least two different glycoproteins in its lipid bilayer is a togavirus, and said at least two different viral glycoproteins are togaviral glycoproteins.

43. The method of claim 42, wherein said togavirus is an alphavirus and said togaviral glycoproteins are alphaviral glycoproteins.

44. A method of introducing a nucleotide sequence into a cell, said method comprising:

transducing a cell permissive for Marburg virus entry with a pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer; said lipid bilayer surrounding said retroviral capsid;

a Marburg virus glycoprotein disposed in said lipid bilayer; and

a desired ribonucleotide sequence.

45. A method of screening agents effective in blocking viral entry into a cell, said method comprising:

(a) treating a pseudotyped retrovirus with said agent, said pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer, said lipid bilayer surrounding said retroviral capsid;

at least two different viral glycoproteins disposed in said lipid bilayer; and

a nucleotide sequence encoding a desired marker, said nucleotide sequence enclosed within said retroviral capsid;

(b) treating a cell permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer with said treated pseudotyped retrovirus; and

(c) identifying eukaryotic cells having the desired marker.

46. The method of claim 45, wherein said virus having at least two different viral glycoproteins disposed in its lipid bilayer is a togavirus and said two different viral glycoproteins are togaviral glycoproteins.

47. The method of claim 46, wherein said togavirus is an alphavirus and said togaviral glycoproteins are alphaviral glycoproteins.

48. The method of claim 45, wherein said agent is an immunological agent.

49. The method of claim 45, wherein said agent is a pharmacological agent.

50. A method of screening agents effective in blocking Marburg virus entry into a cell, said method comprising:

(a) treating a pseudotyped retrovirus with said agent, said pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer, said lipid bilayer surrounding said retroviral capsid;

a Marburg virus glycoprotein disposed in said lipid bilayer; and

a nucleotide sequence encoding a desired marker, said nucleotide sequence enclosed within said retroviral capsid;

(b) treating a cell permissive for Marburg virus entry with said treated pseudotyped retrovirus; and

(c) identifying eukaryotic cells having the desired marker.

51. A method of screening agents effective in blocking viral entry into a cell, said method comprising:

(a) treating a cell permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer with said agent;

(b) contacting said treated cell with a pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer, said lipid bilayer surrounding said retroviral capsid;

at least two different viral glycoproteins disposed in said lipid bilayer;

a nucleotide sequence encoding a desired marker, said nucleotide sequence enclosed within said retroviral capsid; and

(c) identifying eukaryotic cells having the desired marker.

52. A method of screening agents effective in blocking viral entry into a cell, said method comprising:

(a) treating a cell permissive for entry of a Marburg virus with said agent;

(b) contacting said treated cell with a pseudotyped retrovirus having

a retroviral capsid;

a lipid bilayer, said lipid bilayer surrounding said retroviral capsid;

a Marburg virus glycoprotein disposed in said lipid bilayer;

a nucleotide sequence encoding a desired marker, said nucleotide sequence enclosed within said retroviral capsid; and

(c) identifying eukaryotic cells having the desired marker.

53. A kit for forming a pseudotyped retrovirus, said kit comprising:

(a) a first nucleotide sequence encoding a retroviral Gag polypeptide;

(b) a second nucleotide sequence encoding a retroviral Pro polypeptide;

(c) a third nucleotide sequence encoding a retroviral Pol polypeptide; and

(d) a fourth nucleotide sequence encoding at least two different viral glycoproteins.

54. The method of claim 52, wherein said viral glycoproteins are togaviral glycoproteins.

55. A kit for forming a pseudotyped retrovirus, said kit comprising:

(a) a first nucleotide sequence encoding a retroviral Gag polypeptide;

(b) a second nucleotide sequence encoding a retroviral Pro polypeptide;

(c) a third nucleotide sequence encoding a retroviral Pol polypeptide; and

(d) a fourth nucleotide sequence encoding a Marburg virus glycoprotein.

SEQUENCE LISTING

SEQ ID 1: Sequence From plasmid pRR64, similar to that from the Ross River Virus Genome Nucleotides 8380-11330 with the exceptions noted in Kuhn et al., (1991) *Virology* 182:430-431 and in Example 2 herein

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tac ccc tgc tgc tac gaa aaa cag cca gaa cag aca ctg cgg atg ctg gaa gac
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asn val asn arg pro gly tyr tyr glu leu leu glu ala ser met thr cys arg asn arg ser arg

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his arg arg ser val thr glu his phe asn val tyr lys ala thr arg pro tyr

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met arg arg
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SEQ ID 2: Ebola virus Zaire, subtype Mayinga Strain Genome, Genbank Accession Number U23187

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gly asn asn asn thr his his gln asp thr gly glu glu ser ala ser ser gly

aag cta ggc tta att acc aat act att gct gga gtc gca gga ctg atc aca ggc ggg aga aga act
lys leu gly leu ile thr asn thr ile ala gly val ala gly leu ile thr gly gly arg arg thr

cga aga gaa gca att gtc aat gct caa ccc aaa tgc aac cct aat tta cat tac
arg arg glu ala ile val asn ala gln pro lys cys asn pro asn leu his tyr

tgg act act cag gat gaa ggt gct gca atc gga ctg gcc tgg ata cca tat ttc ggg cca gca gcc
trp thr thr gln asp glu gly ala ala ile gly leu ala trp ile pro tyr phe gly pro ala ala

gag gga att tac ata gag ggg cta atg cac aat caa gat ggt tta atc tgt ggg
glu gly ile tyr ile glu gly leu met his asn gln asp gly leu ile cys gly

ttg aga cag ctg gcc aac gag acg act caa gct ctt caa ctg ttc ctg aga gcc aca act gag cta
leu arg gln leu ala asn glu thr thr gln ala leu gln leu phe leu arg ala thr thr glu leu

cgc acc ttt tca atc ctc aac cgt aag gca att gat ttc ttg ctg cag cga tgg
arg thr phe ser ile leu asn arg lys ala ile asp phe leu leu gln arg trp

ggc ggc aca tgc cac att ctg gga ccg gac tgc tgt atc gaa cca cat gat tgg acc aag aac ata
gly gly thr cys his ile leu gly pro asp cys cys ile glu pro his asp trp thr lys asn ile

aca gac aaa att gat cag att att cat gat ttt gtt gat aaa acc ctt ccg gac
thr asp lys ile asp gln ile ile his asp phe val asp lys thr leu pro asp
```

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cag ggg gac aat gac aat tgg tgg aca gga tgg aga caa tgg ata ccg gca ggt att gga gtt aca
gln gly asp asn asp asn trp trp thr gly trp arg gln trp ile pro ala gly ile gly val thr

ggc gtt ata att gca gtt atc gct tta ttc tgt ata tgc aaa ttt gtc ttt tag
gly val ile ile ala val ile ala leu phe cys ile cys lys phe val phe
ttttttcttcagattgcttcatggaaaagctcagcctcaaatcaatgaaaccaggatttaa
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tga
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SEQ ID 3: Marburg virus Genome, Genbank Accession Number Z12132

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atg aag acc aca tgt ttc ctt atc agt ctt atc tta att caa ggg aca aaa aat ctc ccc att tta
Met lys thr thr cys phe leu ile ser leu ile leu ile gln gly thr lys asn leu pro ile leu

gag ata gct agt aat aat caa ccc caa aat gtg gat tcg gta tgc tcc gga act
glu ile ala ser asn asn gln pro gln asn val asp ser val cys ser gly thr

ctc cag aag aca gaa gac gtc cat ctg atg gga ttc aca ctg agt ggg caa aaa gtt gct gat tcc
leu gln lys thr glu asp val his leu met gly phe thr leu ser gly gln lys val ala asp ser

cct ttg gag gca tcc aag cga tgg gct ttc agg aca ggt gta cct ccc aag aat
pro leu glu ala ser lys arg trp ala phe arg thr gly val pro pro lys asn

gtt gag tac aca gag ggg gag gaa gcc aaa aca tgc tac aat ata agt gta acg gat ccc tct gga
val glu tyr thr glu gly glu glu ala lys thr cys tyr asn ile ser val thr asp pro ser gly

aaa tcc ttg ctg tta gat cct cct acc aac atc cgt gac tat cct aaa tgc aaa
lys ser leu leu leu asp pro pro thr asn ile arg asp tyr pro lys cys lys

act atc cat cat att caa ggt caa aac cct cat gca cag ggg atc gcc ctt cat tta tgg gga gca
thr ile his his ile gln gly gln asn pro his ala gln gly ile ala leu his leu trp gly ala

ttt ttt ctg tat gat cgc att gcc tcc aca aca atg tac cga ggc aaa gtc ttc
phe phe leu tyr asp arg ile ala ser thr thr met tyr arg gly lys val phe

gaa ggg aac ata gca gct atg att gtc aat aag aca gtg cac aaa atg att ttc tcg cgg caa gga
thr glu gly asn ile ala ala met ile val asn lys thr val his lys met ile phe ser arg gln

caa ggg tac cgt cat atg aat ctg act tct act aat aaa tat tgg aca agt
gly gln gly tyr arg his met asn leu thr ser thr asn lys tyr trp thr ser

agt aac gga acg caa acg aat gac act gga tgt ttc ggc gct ctt caa gaa tac aat tct aca aag
ser asn gly thr gln thr asn asp thr gly cys phe gly ala leu gln glu tyr asn ser thr lys

aac caa aca tgt gct ccg tcc aaa ata cct cca cca ctg ccc aca gcc cgt ccg
asn gln thr cys ala pro ser lys ile pro pro pro leu pro thr ala arg pro

gag atc aaa ctc aca agc acc cca act gat gcc acc aaa ctc aat acc acg gac cca agc agt gat
glu ile lys leu thr ser thr pro thr asp ala thr lys leu asn thr thr asp pro ser ser asp

gat gag gac ctc gca aca tcc ggc tca ggg tcc gga gaa cga gaa ccc cac aca
asp glu asp leu ala thr ser gly ser gly ser gly glu arg glu pro his thr

act tct gat gcg gtc acc aag caa ggg ctt tca tca aca atg cca ccc act ccc tca cca caa cca
thr ser asp ala val thr lys gln gly leu ser ser thr met pro pro thr pro ser pro gln pro

agc acg cca cag caa gga gga aac aac aca aac cat tcc caa gat gct gtg act
ser thr pro gln gln gly gly asn asn thr asn his ser gln asp ala val thr
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glu leu asp lys asn asn thr thr ala gln pro ser met pro pro his asn thr thr thr ile ser

act aac aac acc tcc aaa cac aac ttc agc act ctc tct gca cca tta caa aac
thr asn asn thr ser lys his asn phe ser thr leu ser ala pro leu gln asn

acc acc aat gac aac aca cag agc aca atc act gaa aat gag caa acc agt gcc ccc tcg ata aca
thr thr asn asp asn thr gln ser thr ile thr glu asn glu gln thr ser ala pro ser ile thr

acc ctg cct cca acg gga aat ccc acc aca gca aag agc acc agc agc aaa aaa
thr leu pro pro thr gly asn pro thr thr ala lys ser thr ser ser lys lys

ggc ccc gcc aca acg gca cca aac acg aca aat gag cat ttc acc agt cct ccc ccc acc ccc agc
gly pro ala thr thr ala pro asn thr thr asn glu his phe thr ser pro pro pro thr pro ser

tcg act gca caa cat ctt gta tat ttc aga aga aag cga agt atc ctc tgg agg
ser thr ala gln his leu val tyr phe arg arg lys arg ser ile leu trp arg

gaa ggc gac atg ttc cct ttt ctg gat ggg tta ata aat gct cca att gat ttt gac cca gtt cca
glu gly asp met phe pro phe leu asp gly leu ile asn ala pro ile asp phe asp pro val pro


aat aca aaa aca atc ttt gat gaa tcc tct agt tct ggt gcc tcg gct gag gaa
asn thr lys thr ile phe asp glu ser ser ser ser gly ala ser ala glu glu

gat caa cat gcc tcc ccc aat att agt tta act tta tct tat ttt cct aat ata aat gag aac act
asp gln his ala ser pro asn ile ser leu thr leu ser tyr phe pro asn ile asn glu asn thr

gcc tac tct gga gaa aat gag aat gat tgt gat gca gag tta aga att tgg agc
ala tyr ser gly glu asn glu asn asp cys asp ala glu leu arg ile trp ser

gtt cag gag gat gac ctg gcc gca ggg ctc agt tgg ata ccg ttt ttt ggc cct gga att gaa gga
val gln glu asp asp leu ala ala gly leu ser trp ile pro phe phe gly pro gly ile glu gly

ctt tac act gct gtt tta att aaa aat caa aac aat ttg gtc tgc agg ttg agg
leu tyr thr ala val leu ile lys asn gln asn asn leu val cys arg leu arg

cgt cta gcc aat caa act gcc aaa tcc ttg gaa ctc tta ttg aga gtc aca act gag gaa aga aca
arg leu ala asn gln thr ala lys ser leu glu leu leu leu arg val thr thr glu glu arg thr

ttc tcc tta atc aat aga cat gct att gac ttt cta ctc aca aga tgg gga gga
phe ser leu ile asn arg his ala ile asp phe leu leu thr arg trp gly gly

aca tgc aaa gtg ctt gga cct gat tgt tgc atc ggg ata gaa gac ttg tcc aaa aat att tca gag
thr cys lys val leu gly pro asp cys cys ile gly ile glu asp leu ser lys asn ile ser glu

caa att gac caa att aaa aag gac gaa caa aaa gag ggg act ggt tgg ggt ctg
gln ile asp gln ile lys lys asp glu gln lys glu gly thr gly trp gly leu

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gly gly lys trp trp thr ser asp trp gly val leu thr asn leu gly ile leu leu leu leu ser

ata gct gtc ttg att gct cta tcc tgt att tgt cgt atc ttt act aaa tat atc
ile ala val leu ile ala leu ser cys ile cys arg ile phe thr lys tyr ile

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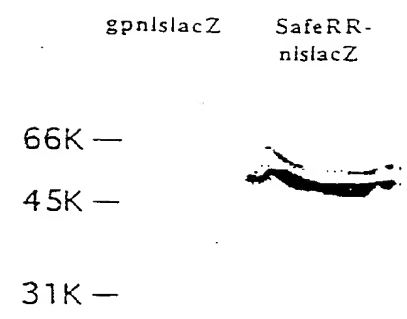

FIG. 1

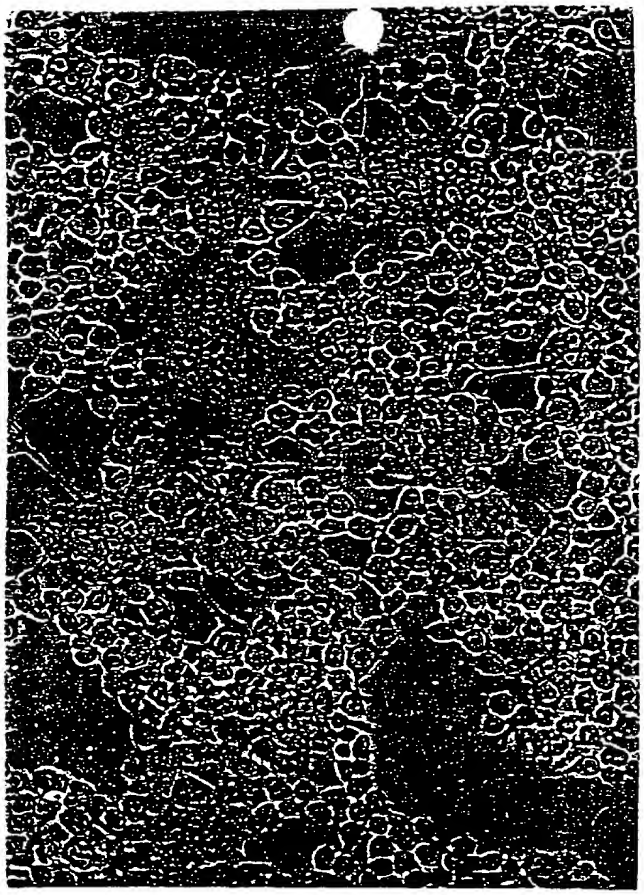

Fig 2 A

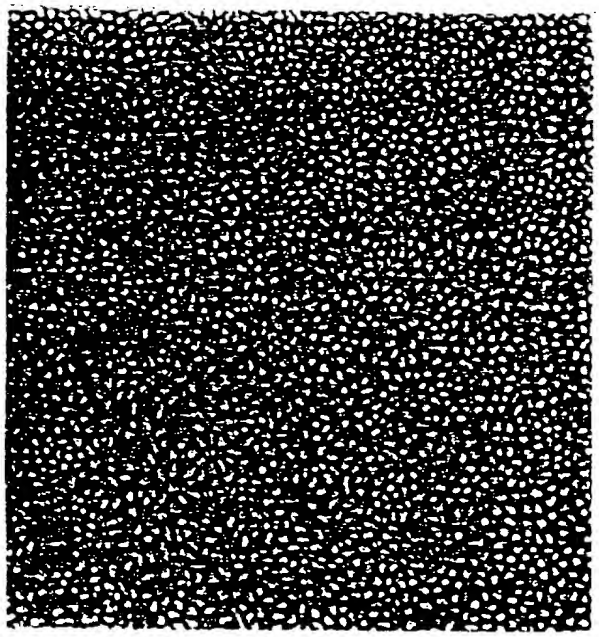

Fig. 2 B

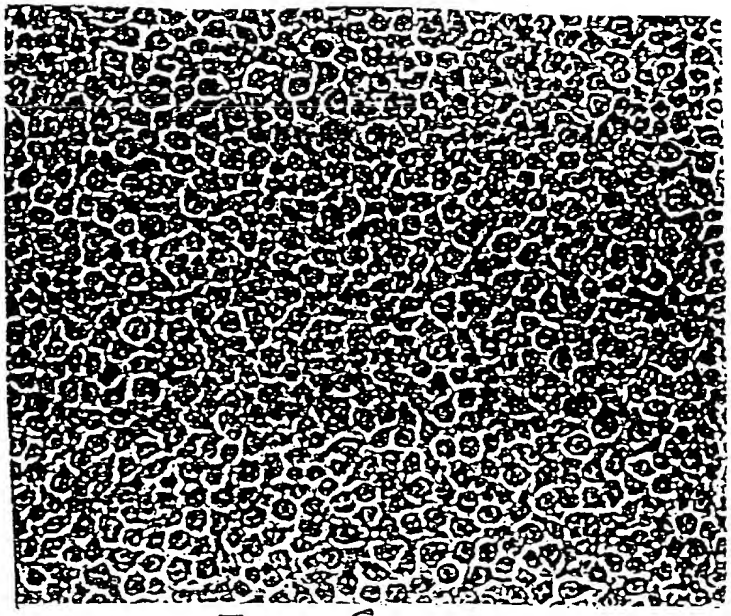

Fig. 2 C

Fig. 3 A

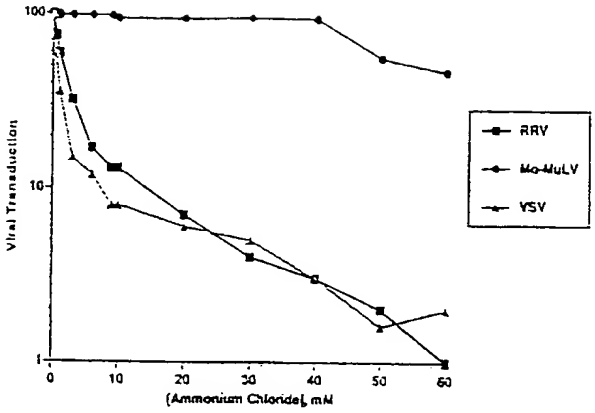

Fig. 3 B

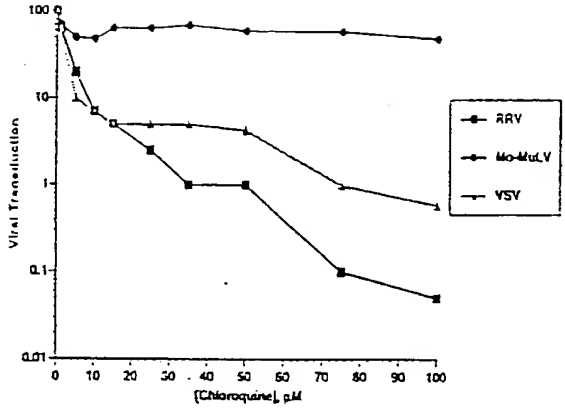

Fig 4 A

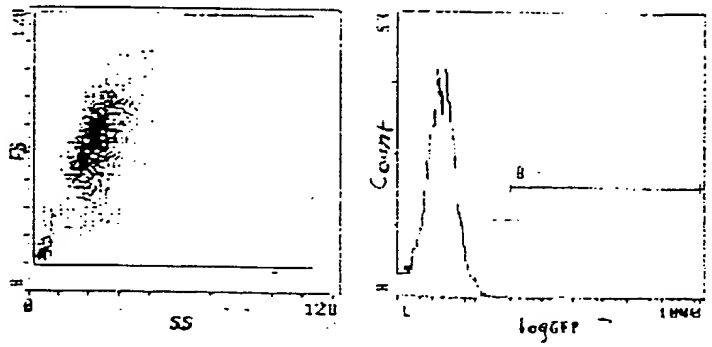

Fig 4 B

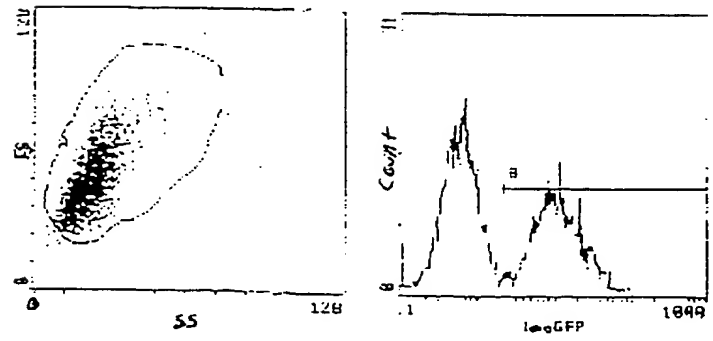

Fig5 A

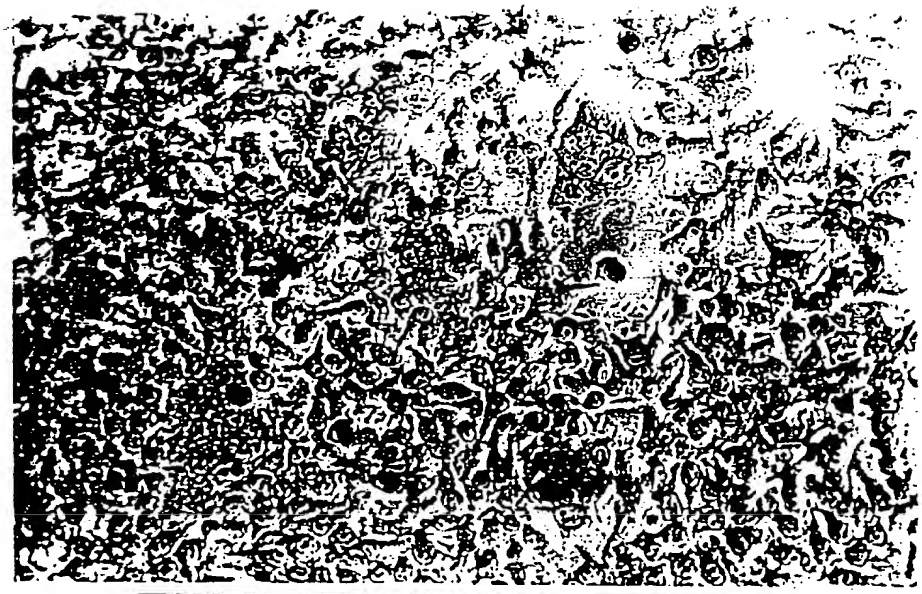

Fig. 5B